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PATENT & TRADEMARK OFFICE

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Dean L. Engelhardt et al.	
Serial No. 09/727,349	Group Art Unit:1655
Filed: November 30, 2000	Examiner: Arun Chakrabarti, Ph.D.
Title: NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES	

527 Madison Avenue, 9th Floor
New York, NY 10022-4304
November 20, 2003

FILED BY EXPRESS MAIL

Mail Stop Petition
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PETITION UNDER 37 C.F.R. §1.137(b) TO REVIVE AN UNINTENTIONALLY ABANDONED APPLICATION

Dear Sirs:

Applicants submit this Petition to the Commissioner under the provisions of 37 C.F.R. §1.137(b) to revive the above-identified application in which taking action was unintentionally delayed. A response to the previously issued December 12, 2002 Office Action was originally due on March 12, 2003. Upon the expected granting of this Petition, the accompanying response in the form of a Request For Continuation Application Under 37 C.F.R. §1.53(b) will be considered as having been timely filed.

11/24/2003 SLUANG1 00000033 051135 09727349
01 FC:1453 1330.00 DA

EXPRESS MAIL CERTIFICATE

"Express Mail" Label No. EV160376692US
Deposit Date November 20, 2003
I hereby certify that this paper and the attachments herein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington DC 20231.

Ronald C. Fedus
Reg. No. 32,567

November 20, 2003
Date

TERMINAL DISCLAIMER TO ACCOMPANY PETITION (Period of disclaimer to be completed by Petitions Examiner)	Docket Number (Optional) ENZ-52(D1)(C2)

In re Application of:
Name: Dean L. Engelhardt et al.

Application Number: 09/727,349

Filed: November 30, 2000

For: **NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES**

The owner*, Enzo Life Sciences, Inc. (formerly named "Enzo Diagnostics, Inc.") of 100 percent interest in the above-identified application hereby disclaims a terminal part of the term of any patent granted the above-identified application equivalent to: (1) if the above-identified application is a design application, the period of abandonment of the above-identified application, and (2) if the above-identified application is a utility or plant application, the lesser of: (a) the period of abandonment of the application; or (b) the period extending beyond twenty years from the date on which the above-identified application was filed in the United States or, if the application contains a specific reference to an earlier filed application(s) under 35 U.S.C. 120, 121, or 365(c), from the date on which the earliest such application was filed. This disclaimer also applies to any patent granted on a utility or plant application filed before June 8, 1995, or a design application, that contains a specific reference under 35 U.S.C. 120, 121, or 365(c) to the above-identified application. This disclaimer is binding upon the grantee, and its successors or assigns.

Check either box 1 or 2 below, if appropriate.

1. /__/ For submissions on behalf of an organization (e.g. corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

2. /X/ The undersigned is an attorney of record.

Signature — November 20, 2003 (Date)
Reg. No. 32,567
Ronald C. Fedus
Typed or printed name

/X/ The Patent and Trademark Office is hereby authorized to charge Deposit Account No. 05-1135 for the Terminal Disclaimer fee Under 37 C.F.R. 1.20(d) and for any other fees required.

/_/ Terminal disclaimer fee under 37 CFR 1.20(d) included.

* Certification under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner). Form PTO/SB/96 may be used for making this certification. See MPEP ∋ 324.

THE STATEMENT BELOW IS FOR OFFICE USE ONLY

In accordance with the decision granting the petition filed on _____, this terminal disclaimer is accepted. The period of abandonment specified above has been accepted as equivalent to _____ months.

11/26/2003 SLUANG1 00000001 051135 09727349

01 FC:1814 110.00 DA

_____
Petitions Examiner

Attorney's Docket: Enz-52(D1)(C2)

The above-identified application became unintentionally abandoned after March 12, 2003, which was the date that a response to the December 12, 2002 Office Action was originally due. A Notice of Abandonment was mailed on September 23, 2003 (copy attached as Exhibit 1).

It is hereby requested that this application be revived because the entire delay in filing the response to the December 12, 2002 Office Action until the filing of this Petition was unintentional. Because this Petition is being filed within three months of the September 23, 2003 mailing date of the Notice of Abandonment, no terminal disclaimer is believed to be required. In the alternative that such is required, Applicants are providing a Terminal Disclaimer attached as Exhibit 2.

As indicated above, a response to the December 12, 2002 Office Action in the form of a Request For Continuation Application Under 37 C.F.R. §1.53(b) is being submitted concurrently herewith and is attached as Exhibit 3. Applicants' Continuation Request (Exhibit 3) also includes a Preliminary Amendment.

The fee for filing a Petition to Revive an Unintentionally Abandoned Application Under 37 C.F.R. §1.137(b) is $665.00 for a small entity. Small entity status was previously established in this application and is still applicable. The Patent and Trademark Office is hereby authorized to charge Deposit Account No. 05-1135 for the requisite large entity fee of $665.00. The Patent and Trademark Office is further authorized hereby to charge Deposit Account No. 05-1135 for any other fees required in connection with this Petition, the attached Amendment (Exhibit 2), or Terminal Disclaimer (Exhibit 1).

A duplicate copy of this Petition but without attached Exhibits 1-3 is also submitted herewith.

Favorable action on this Petition is earnestly solicited.

Respectfully submitted,

Ronald C. Fedus
Registration No. 32,567
Attorney for Applicants

ENZO LIFE SCIENCES, INC.
c/o Enzo Biochem, Inc.
527 Madison Avenue (9th Fl.)
New York, New York 10022
Telephone: (212) 583-0100
Fax: (212) 583-0150

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/727,349	11/30/2000	Dean L. Engelhardt	Enz-52 (D1)	3143

28170 7590 09/23/2003

ENZO DIAGNOSTICS, INC.
C/O ENZO BIOCHEM INC.
527 MADISON AVENUE 9TH FLOOR
NEW YORK, NY 10022

EXAMINER
CHAKRABARTI, ARUN K

ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 09/23/2003

COPY

Please find below and/or attached an Office communication concerning this application or proceeding.

Notice of Abandonment

Application No.	Applicant(s)
09/727,349	Engelhardt
Examiner	**Art Unit**
Arun Chakrabarti	1634

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

This application is abandoned in view of:

1. ☒ Applicant's failure to timely file a proper reply to the Office letter mailed on *Dec 6, 2002* .

   (a) ☐ A reply was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply (including a total extension of time of ___ month(s)) which expired on _____ .

   (b) ☐ A proposed reply was received on _____ , but it does not constitute a proper reply under 37 CFR 1.113(a) to the final rejection.

   (A proper reply under 37 CFR 1.113 to a final rejection consists only of: (1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114).

   (c) ☐ A reply was received on _____ but it does not constitute a proper reply, or a bona fide attempt at a proper reply, to the non-final rejection. See 37 CFR 1.85(a) and 1.111. (See explanation in box 7 below).

   (d) ☒ No reply has been received.

2. ☐ Applicant's failure to timely pay the required issue fee and publication fee, if applicable, within the statutory period of three months from the mailing date of the Notice of Allowance (PTOL-85).

   (a) ☐ The issue fee and publication fee, if applicable, was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the statutory period for payment of the issue fee (and publication fee) set in the Notice of Allowance (PTOL-85).

   (b) ☐ The submitted issue fee of $_____ is insufficient. A balance of $_____ is due.

   The issue fee required by 37 CFR 1.18 is $_____ . The publication fee, if required by 37 CFR 1.18(d) is $_____

   (c) ☐ The issue fee and publication fee, if applicable, has not been received.

3. ☐ Applicant's failure to timely file corrected drawings as required by, and within the three-month period set in, the Notice of Allowability (PTO-37).

   (a) ☐ Proposed new formal drawings were received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply.

   (b) ☐ No corrected drawings have been received.

4. ☐ The letter of express abandonment which is signed by the attorney or agent of record, the assignee of the entire interest, or all of the applicants.

5. ☐ The letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34(a)) upon the filing of a continuing application.

6. ☐ The decision by the Board of Patent Appeals and Interferences rendered on _____ and because the period for seeking court review of the decision has expired and there are no allowed claims.

7. ☐ The reason(s) below:

GARY BENZION, PH.D
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

Petitions to revive under 37 CFR 1.137(a) or (b), or requests to withdraw the holding of abandonment under 37 CFR 1.181, should be promptly filed to minimize any negative effects on patent term.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Anticipated Classification Of this Application:
Class _____ Subclass _____

Prior Application:
Exam'r: **Arun K. Chakrabarti, Ph.D.**
Prior Group Art Unit: **1634**

Attorney's Docket No.:
Enz-52(D1)(C2)

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Mail Stop Patent Application
Commissioner for Patents
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Alexandria, VA 22313-1450

COPY

Sir:

This is a request for filing a __X__ CONTINUATION ___ DIVISIONAL ___ CONTINUATION-IN-PART application under ___ 37 CFR §1.53(b) __X__ 37 CFR §1.53(b), of pending prior application Serial No. 09/727,349 filed on November 30, 2000 of Engelhardt, et al.
Inventor(s)

for

NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES
Title of Invention

1. __X__ Enclosed is a copy of the prior application, as originally filed and an affidavit or declaration verifying it as a true copy.

2. __X__ Small entity status was previously established in a prior application and is still applicable.

EXPRESS MAIL CERTIFICATE

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Ronald C. Fedus
Reg. No. 32,567

November 20, 2003
Date

---

3. X The filing fee is calculated as follows:

CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT									
					RATE			FEE	
	Number Filed		Number Extra		Small Entity	Other Entity		Small Entity	Other Entity
Total Claims	30	=	10	x	$ 9	$ N/A	=	$90	$ ---
Independent Claims	1	=	0	x	$ 43	$ N/A	=	$ 0	$ ---
Multiple Dependent Claims Presented: ____Yes X No					$135	$270		$ --	$ ---
* If the difference in Col. 1 is less than zero, enter "0" in Col.2						BASIC FEE		$385	$ ---
						TOTAL FEE		$475	$ ---

4. X The Commissioner is hereby authorized to charge payment of the filing fee as well as the following fees associated with this application or to credit any overpayment to Deposit Account No. 05-1135. A **duplicate** copy of this sheet is enclosed.

X Any additional filing fees required under 37 CFR §1.16.

X Any patent application processing fees under 37 CFR §1.17.

5. X The Commissioner is hereby authorized to charge payment of the following fees during the pendency of this application or credit any overpayment to Deposit Account No. 05-1135. A **duplicate** copy of this sheet is enclosed.

X Any patent application processing fees under 37 CFR §1.17.

____ The issue fee set in 37 CFR §1.18 at or before mailing of the Notice of Allowance, pursuant to 37 CFR §1.311(b).

_____ Any filing fees under 37 CFR §1.16 for presentation of extra claims.

6. _____ A check in the amount of $_____ is enclosed.

7. ___X___ Cancel claims ___2-90___.

8. ___X___ Amend the specification by inserting before the first line of the sentence: ---- This is a ___X___ continuation, _____ divisional of U.S. Patent Application Serial No. 09/727,349, filed on November 30, 2000, which is a continuation of U.S. Patent Application Serial No. 09/302,818 filed on February 3, 1998, now abandoned. ---

9. _____ Transfer the drawings from the prior application.

10. ___X___ Informal ___X___ Formal _____ drawings are enclosed.

11. _____ Abandon said prior application as of the filing date accorded this application. A **duplicate** copy of this sheet is enclosed for filing in the prior application file.

12. _____ Priority of application Serial No. _____ filed on _____ is claimed under 35 U.S.C. §119. _____ The certified copy of the priority application has been filed in prior application Serial No. _____, filed _____.

13. ___X___ The prior application is assigned of record to Enzo Diagnostics, Inc., c/o Enzo Biochem, Inc., 527 Madison Avenue, New York, New York 10022. A copy of the Assignment is enclosed.

14. ___X___ A preliminary amendment is enclosed.

15. ___X___ The power of attorney in the prior application is to: Ronald C. Fedus, Reg. No. 32,567.

(a) ___X___ The power appears in the original papers in the prior application.

(b) _____ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

(c) _____ A copy of a new substitute power of attorney is enclosed.

(d) _____ A copy of a new Associate Power of Attorney is enclosed.

(e) ___X___ Address all future communications to: (May only be completed by applicant, or attorney or agent of record.)

Ronald C. Fedus, Esq.
Enzo Life Sciences, Inc.
c/o Enzo Biochem, Inc.
527 Madison Avenue (9th Floor)
New York, New York 10022

16. ___X___ Oath or Declaration

(a)_____ Newly executed (original or copy)

(b) ___X___ Copy from a prior application (37 CFR 1.63(d))

(c) ___X___ With Power of Attorney

(D) _____ Without Power of Attorney

17. ___X___ I hereby verify that the attached papers are a true copy of prior application Serial No. 09/727,349, filed on November 30, 2000.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

November 20, 2003
Date

Respectfully submitted,

Ronald C. Fedus
Registration No. 32,567
Attorney for Applicants
_____ Inventor(s)
_____ Assignee of Complete Interest
__X__ Attorney or Agent of Record
__X__ Filed under 37 CFR § 1.34(a)

Address of Signator:
Enzo Life Sciences, Inc.
c/o Enzo Biochem, Inc.
527 Madison Avenue (9th Floor)
New York, NY 10022-4304

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Anticipated Classification Of this Application:
Class _____ Subclass _____

Prior Application:
Exam'r: **Arun K. Chakrabarti, Ph.D.**
Prior Group Art Unit: **1634**

Attorney's Docket No.:
Enz-52(D1)(C2)

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Reg. N . 32,567

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Ronald C. Fedus
Registration No. 32,567
Attorney for Applicants
_____ Inventor(s)
_____ Assignee of Complete Interest
__X__ Attorney or Agent of Record
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Address of Signator:
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c/o Enzo Biochem, Inc.
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New York, NY 10022-4304

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Anticipated Classification Of this Application: Class ____ Subclass ____	Prior Application: Exam'r: **Arun K. Chakrabarti, Ph.D.** Prior Group Art Unit: **1634**	Attorney's Docket No.: **Enz-52(D1)(C2)**

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c/o Enzo Biochem, Inc.
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November 20, 2003
Date

Respectfully submitted,

Ronald C. Fedus
Registration No. 32,567
Attorney for Applicants
_____ Inventor(s)
_____ Assignee of Complete Interest
__X__ Attorney or Agent of Record
__X__ Filed under 37 CFR § 1.34(a)

Address of Signator:
Enzo Life Sciences, Inc.
c/o Enzo Biochem, Inc.
527 Madison Avenue (9th Floor)
New York, NY 10022-4304

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Engelhardt et al.	
Continuation of Serial No. 09/727,349	Group Art Unit: Not Yet Known
Filed: November 30, 2000	Examiner: Not Yet Known
Title: **NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES**	**Previous Group Art Unit: 1634** **Prev. Ex'r: Arun K. Chakrabarti, Ph.D.**

527 Madison Avenue (9th Floor)
New York, NY 10022-4304
November 20, 2003

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PRELIMINARY AMENDMENT

(ACCOMPANYING CONTINUATION APPLICATION UNDER 37 C.F.R. §1.53(b))

Dear Sirs:

Prior to examination, please amend the above-identified application as follows:

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Ronald C. Fedus
Reg. No. 32,567

November 20, 2003
Date

PLEASE AMEND THIS APPLICATION AS FOLLOWS:

In The Title:

Change the title of the invention to:

-- *In vitro* Processes for Producing Multiple Copies of Primer Sequence-Free Specific Nucleic Acid Independent of Intermediate Structure -- .

In The Claims:

Please cancel claim 1.

Please add new claims 91-120.

Claim 1 (Canceled Hereinabove)

Claims 2-90 (Canceled in Continuation Request)

91. (NEW) An *in vitro* process for producing more than one copy of a specific nucleic acid, said process being independent of a requirement for the formation of an intermediate structure by the introduction of a promoter sequence or an asymmetric restriction site for the production of said specific nucleic acid, said process comprising the steps of:

(a) providing a nucleic acid sample containing or suspected of containing the sequence of said specific nucleic acid;

(b) contacting said sample with a mixture comprising:

(i) nucleic acid precursors,

(ii) one or more specific nucleic acid primers each of which is complementary to a distinct sequence of said specific nucleic acid, and

(iii) an effective amount of a nucleic acid producing catalyst; and

(c) allowing said mixture to react under isostatic conditions of temperature, buffer and ionic strength to produce more than one copy of said specific nucleic acid.

92. (NEW) The process of claim 91, wherein said specific nucleic acid is single-stranded or double-stranded.

93. (NEW) The process of claim 91, wherein said specific nucleic acid comprises deoxyribonucleic acid, ribonucleic acid, a DNA.RNA hybrid, a polymer capable of acting as a template for a nucleic acid polymerizing catalyst, or a combination of any of the foregoing.

94. (NEW) The process of claim 91, wherein said specific nucleic acid is in solution.

95. (NEW) The process of claim 94, further comprising the step of treating said specific nucleic acid with a blunt-end promoting restriction enzyme.

96. (NEW) The process of claim 91, wherein said specific nucleic acid is isolated or purified prior to the contracting step (b) or the reacting step (c).

97. (NEW) The process of claim 96, wherein said isolation or purification of said specific nucleic acid is carried out by means of sandwich or sandwich capture.

98. (NEW) The process of claim 97, further comprising the step of releasing said captured specific nucleic acid.

99. (NEW) The process of claim 98, wherein said releasing step is carried out by means of a restriction enzyme.

100. (NEW) The process of claim 91, wherein said nucleic acid precursors comprise nucleoside triphosphates, nucleoside trisphosphate analogs, or a combination thereof.

101. (NEW) The process of claim 100, wherein said nucleoside triphosphates comprise deoxyadenosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, deoxythymidine 5'-triphosphate, deoxycytidine 5'-triphosphate, adenosine 5'-triphosphate, guanosine 5'-triphosphate, uridine 5'-triphosphate and cytidine 5'-triphosphate, or a combination of any of the foregoing.

102. (NEW) The process of claim 100, wherein said nucleoside triphosphate analogs are naturally occurring or synthetic, or a combination thereof.

103. (NEW) The process of claim 100, wherein at least one of said nucleoside triphosphates or nucleoside triphosphate analogs is modified on the sugar, phosphate or base.

104. (NEW) The process of claim 100, wherein said specific nucleic acid primers comprise deoxyribonucleic acid, ribonucleic acid, a DNA.RNA copolymer, a polymer

capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization, or a combination of any of the foregoing.

105. (NEW) The process of claim 91, wherein said specific nucleic acid primers comprise oligo- or polynucleotides.

106. (NEW) The process of claim 91, wherein said specific nucleic acid primers comprise a 3'-hydroxyl group or an isosteric configuration of heteroatoms.

107. (NEW) The process of claim 106, wherein said heteroatoms comprise nitrogen or sulfur.

108. (NEW) The process of claim 91, wherein said specific nucleic acid primers are not substantially complementary to one another.

109. (NEW) The process of claim 108, wherein said specific nucleic acid primers contain no more than five complementary base-pairs in the sequences therein.

110. (NEW) The process of claim 91, wherein said specific nucleic acid primers comprise from about 5 to about 100 nucleotides.

111. (NEW) The process of claim 110, wherein said specific nucleic acid primers comprise from about 8 to about 20 nucleotides.

112. (NEW) The process of claim 91, wherein said specific nucleic acid primers comprise at least one non-complementary nucleotide or nucleotide analog base, or at least one sequence thereof.

113. (NEW) The process of claim 112, wherein said specific nucleic acid primers further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs.

114. (NEW) The process of claim 113, wherein said noncomplementary nucleotide or nucleotide analogs in said specific nucleic acid primers comprise from about 5 to about 20 nucleotides.

115. (NEW) The process of claim 112, wherein said noncomplementary base sequence or sequences are linked together by other than a phosphodiester bond.

116. (NEW) The process of claim 91, wherein said nucleic acid producing catalyst comprises DNA polymerase, reverse transcriptase, or both.

117. (NEW) The process of claim 91, wherein siad nucleic precursors or said specific primers or both are modified by at least one intercalating agent.

118. (NEW) The process of claim 91, further comprising the step (d) of detecting the product produced in step (c).

119. (NEW) The process of claim 118, wherein said detecting step (d) is carried out by means of incorporating into the product a labeled primer, a labeled precursor, or a combination thereof.

120. (NEW) The process of claim 91, further comprising the step of regenerating said one or more specific nucleic acid primers.

* * * * * * *

REMARKS

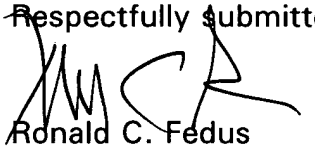
This application is a continuation application of U.S. Patent Application Serial No. 09/727,349, filed on November 30, 2000.

In this application, new claims 91-120 have been added in place of claim 1, the latter now having been canceled above. The above added claims 91-120 correspond in large part to claims 1-30, which latter claims were pending and canceled in the parent, U.S. Patent Application Serial No. 09/727,349, filed November 30, 2000.

The fee for adding new claims 91-120 is $90 based upon the presentation of 10 claims above the 20 claims paid for in the basic filing fee [10 claims X $9 = $90]. The United States Patent and Trademark Office is hereby authorized to charge the requisite $90 claim fee to Deposit Account No. 05-1135. No other fee or fees are believed due in connection with this continuation request. In the event that any other fee or fees are due, however, authorization is hereby given to charge the amount of any such fee(s) to Deposit Account No. 05-1135, or to credit any overpayment thereto.

Early and favorable action is respectfully requested.

Respectfully submitted,



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Attorney's Docket No.: Enz-52

APPLICATION

FOR

UNITED STATES LETTERS PATENT

To all Whom it may concern:

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Ronald C. Fedus
Reg. No. 32,567

NOV 20 2003
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Be it known that

Dean L. Engelhardt,
Jannis G. Stavrianopoulos,
James J. Donegan &
Elazar Rabbani

have invented certain new and useful improvements in

NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES

of which the following is a full, clear and exact description.

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JANUARY 13, 1994
Date

Attorney Docket No.: Enz-52

NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES

FIELD OF THE INVENTION

This invention relates to the field of *in vitro* and *in vivo* production of nucleic acid production and to nucleic constructs and protein-nucleic acid conjugates for use in such production.

All patents, patent publications, scientific articles, and videocassettes cited or identified in this application are hereby incorporated by reference in their entirety in order to describe more fully the state of the art to which the present invention pertains.

BACKGROUND OF THE INVENTION

Current methodology cited heretofore in the literature relating to amplification of a specific target nucleic acid sequence *in vitro* essentially involve 2 distinct elements:

1. repeated strand separation or displacement or a specific "intermediate" structure such as a promoter sequence linked to the primer or introduction an assymetric restrictrion site not originally present in the nucleic acid target; followed by

2. production of nucleic acid on the separated strand or from an "intermediate" structure.

Separation can be accomplished thermally or by enzymatic means. Following this separation, production is accomplished enzymatically using the separated strands as templates.

Of the established amplification procedures, Polymerase Chain Reaction (PCR) is the most widely used. This procedure relies on thermal strand separation, or reverse transcription of RNA strands followed by thermal dissociation. At least one primer per strand is used and in each cycle only one copy per separated strand is produced. This procedure is complicated by the requirement for cycling equipment, high reaction temperatures and specific thermostable enzymes. (Saiki, et al., Science 230:1350-1354 (1985); Mullis and Faloona, Methods in Enzymology 155: 335-351 (1987); U.S. Patent Nos. 4,683,195 and 4,883,202).

Other processes, such as the Ligase Chain Reaction (LCR) (Backman, K., European Patent Application Publication No. 0 320 308; Landegren, U., et al. Science 241 1077 (1988); Wu, D. and Wallace, R.B. Genomics 4 560 (1989); Barany, F. Proc. Nat. Acad. Sci USA 88:189 (1991)), and Repair Chain Ligase Reaction (RLCR) or Gap Ligase Chain Reaction (GLCR) (Backman, K. et al. (1991) European Patent Application Publication No. 0 439 182 A; Segev, D. (1991) European Patent Application Publication No. 0 450 594) also use repeated

thermal separation of the strands and each cycle produces only one ligated product. These procedures are more complicated than PCR because they require the use of an additional thermostable enzyme such as a ligase.

More complicated procedures are the Nucleic Acid Sequence Based Amplification (NASBA) and Self Sustained Sequence Reaction (3SR) amplification procedures. (Kwoh, D.Y. et al., Proc Nat Acad Sci., USA., 86:1173-1177 (1989); Guatelli,J.C. et al., 1990 Proc Nat Acad Sci., USA 87:1874-1878 (1990) and the Nucleic Acids Sequence Based Amplification (NASBA) (Kievits,T., et al J. Virol. Methods 35:273-286 (1991); and Malek, L.T., U.S. Patent No. 5,130,238). These procedures rely on the formation of a new "intermediate" structure and an array of different enzymes, such as reverse transcriptase, ribonuclease H, T7 RNA polymerase or other promotor dependant RNA polymerases and they are further disadvantaged by the simultaneous presence of ribo- and deoxyribonucleotide tripohsphates precursors.

For the intermediate construct formation, the primer must contain the promotor for the DNA dependent RNA polymerase. The process is further complicated because the primer is, by itself, a template for the RNA polymerase, due to its single-stranded nature.

The last of the major amplification procedures is Strand Displacement Amplification (SDA) (Walker, G.T. and Schram, J.L., European Patent Application Publication No. 0 500 224 A2; Walker, G.T.

et al. European Patent Application No. 0 543 612 A2; Walker, G.T., European Patent Application Publication No. 0 497 272 A1; Walker, G.T. et al., Proc Natl Acad Sci USA 89:392-396 (1992); and Walker, G.T. et al., Nuc Acids Res. 20:1691-1696 (1992)). The intermediate structure of this procedure is formed by the introduction of an artificial sequence not present in the specific target nucleic acid and which is required for the assymetric recognition site of the restriction enzyme. Again this procedure involves more than one enzyme and the use of thio nucleotide triphosphate precursors in order to produce this assymetric site necessary for the production step of this amplification scheme.

The random priming amplification procedure (Hartley, J.L., U.S. Patent No. 5,043,272) does not relate to specific target nucleic acid amplification.

Probe amplification systems have been disclosed which rely on either the amplification of the probe nucleic acid or the probe signal following hybridization between probe and target. As an example of probe amplification is the Q-Beta Replicase System (QB) developed by Lizardi and Kramer and their colleagues. QB amplification is based upon the RNA-dependent RNA polymerase derived from the bacteriophage QB. This enzyme can synthesize large quantities of product strand from a small amount of template strand, roughly on the order of 10^6 to 10^9 (million to billion) increases. The QB replicase system and its replicatable RNA probes are described by Lizardi et al., "Exponential amplification of recombinant RNA hybridization probes,"

Biotechnology 6:1197-1202 (1988); Chu et al., U.S. Patent No. 4,957,858; and well as by Keller and Manak (DNA Probes, MacMillan Publishers Ltd, Great Britain, and Stockton Press (U.S. and Canada, 1989, pages 225-228). As discussed in the latter, the Qß replicase system is disadvantaged by non-specific amplification, that is, the amplification of non-hybridized probe material, which contributes to high backgrounds and low signal-to-noise ratios. Such attendent background significantly reduces probe amplification from its potential of a billion-fold amplification to something on the order of 10^4 (10,000 fold). In addition, the Q beta amplification procedure is a signal amplification - and not a target amplification.

In vivo

Literature covering the introduction of genes or antisense nucleic acids into a cell or organism is very extensive (Larrick, J.W. and Burck, K. Gene Therapy Elsevier Science Publishing Co., Inc, New York (1991); Murray, J.A.H. ed Antisense RNA and DNA, Wiley-Liss, Inc., New York (1992)). The biological function of these vectors generally requires inclusion of at least one host polymerase promoter.

The present invention as it relates to *in vitro* and *in vivo* production of nucleic acids is based on novel processes, constructs and conjugates which overcome the complexity and limitations of the above-mentioned documents.

SUMMARY OF THE INVENTION

The present invention provides an *in vitro* process for producing more than one copy of a specific nucleic acid in which the process is independent of any requirement for the introduction of an intermediate structure for the production of the specific nucleic acid. The process comprises three steps, including (a) providing a nucleic acid sample containing or suspected of containing the sequence of the specific nucleic acid; (b) contacting the sample with a three component reaction mixture; and (c) allowing the mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing more than one copy of the specific nucleic acid. The reaction mixture comprises: (i) nucleic acid precursors, (ii) one or more specific nucleic acid primers each of which is complementary to a distinct sequence of the specific nucleic acid, and (iii) an effective amount of a nucleic acid producing catalyst.

In another aspect, the present invention provides an *in vitro* process for producing more than one copy of a specific nucleic acid in which the products are substantially free of any primer-coded sequences. Such a process comprises the following steps, including (a) providing a nucleic acid sample containing or suspected of containing the sequence of the specific nucleic acid; (b) contacting the sample with a three component mixture (the mixture comprising (i) nucleic acid precursors, (ii) one or more specific polynucleotide primers comprising at least one ribonucleic acid segment each of which primer

is substantially complementary to a distinct sequence of the specific nucleic acid, and (iii) an effective amount of a nucleic acid producing catalyst); and (c) allowing the mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing at least one copy of the specific nucleic acid; and (d) removing substantially or all primer-coded sequences from the product produced in step (c). By removing such sequences, a primer binding site is regenerated, thereby allowing a new priming event to occur and producing more than one copy of the specific nucleic acid.

The present invention also provides an *in vitro* process for producing more than one copy of a specific nucleic acid in which the products are substantially free of any primer-coded sequences. In the steps of this process, said process comprising a nucleic acid sample containing or suspected of containing the sequence of the specific nucleic acid is provided, and contacted with a reaction mixture. The mixture comprises (i) unmodified nucleic acid precursors, (ii) one or more specific chemically-modified primers each of which primer is substantially complementary to a distinct sequence of said specific nucleic acid, and (iii) an effective amount of a nucleic acid producing catalyst. The mixture thus contacted is allowed to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing at least one copy of the specific nucleic acid. In a further step, substantially or all primer-coded sequences from the product produced in the reacting step is removed to regenerate a primer binding site. The regeneration of a primer binding site thereby allows a new

priming event to occur and the production of more than one copy of said specific nucleic acid.

An additional provision of the present invention is an *in vitro* process for producing more than one copy of a specific nucleic acid in which the products are substantially free of any primer-coded sequences. In this instance, the process comprises the steps of: (a) providing a nucleic acid sample containing or suspected of containing the sequence of the specific nucleic acid; and (b) contacting the sample with a reaction mixture (the mixture comprising (i) unmodified nucleic acid precursors, (ii) one or more specific unmodified primers comprising at least segment each of which primer comprises at least one non-complementary sequence to a distinct sequence of the specific nucleic acid, such that upon hybridization to the specific nucleic acid, at least one loop structure is formed, and (iii) an effective amount of a nucleic acid producing catalyst). The mixture so formed is allowed to react in step (c) under isostatic conditions of temperature, buffer and ionic strength, thereby producing at least one copy of the specific nucleic acid; which step is followed by (d) removing substantially or all primer-coded sequences from the product produced in step (c) to regenerate a primer binding site. The regeneration of a primer binding site thereby allows a new priming event to occur and the production of more than one copy of said specific nucleic acid.

Another embodiment of the present invention concerns a promoter-independent non-naturally occurring nucleic acid construct

which when present in a cell produces a nucleic acid without the use of any gene product coded by the construct.

In yet another embodiment, the present invention provides a conjugate comprising a protein-nucleic acid construct in which the nucleic acid construct does not code for said protein, and which conjugate produces a nucleic acid when present in a cell.

The present invention also has significant *in vivo* applications. In one such application, an *in vivo* process is provided for producing a specific nucleic acid. The *in vivo* process comprises the steps of (a) providing a conjugate comprising a protein-nucleic acid construct, the conjugate being capable of producing a nucleic acid when present in a cell; and (b) introducing such a conjugate into a cell, thereby producing the specific nucleic acid.

Another significant aspect of the present invention relates to a construct comprising a host promoter located on the construct such that the host transcribes a sequence in the construct coding for a different RNA polymerase, which after translation is capable of recognizing its cognate promoter and transcribing from a DNA sequence of interest from the construct with the cognate promoter oriented such that it does not promote transcription from the construct of the different RNA polymerase.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (A-F) depicts various nucleic acid construct forms contemplated by the invention in which at least one single-stranded region are located therein.

Figure 2 (A-F) depicts the functional forms of the nucleic acid constructs illustrated in Figure 1 (A-F).

Figure 3 (A-C) is an illustration of three nucleic acid constructs with an RNA polymerase covalently attached to a transcribing cassette.

Figure 4 (A-C) illustrates three nucleic acid constructs with promoters for endogenous RNA polymerase.

Figure 5 is a nucleic acid sequence for M13mp18.

Figure 6 shows the sequence and the positions of the primers derived from M13mp18 which were employed in the present invention for nucleic acid production.

Figure 7 illustrates appropriate restriction sites in M13mp18.

Figure 8 is an agarose gel with a lane legend illustrating the experimental results in Example 5 in which amplification of the M13 fragment was carried out in the presence of a large excess (1500 fold) of irrelevant DNA.

Figure 9 is an agarose gel with a lane legend illustrating the results in Example 8 in which the effect of variations of reaction conditions on the product obtained in Example 3 was investigated.

Figure 10 is an agarose gel with a lane legend that illustrates the results of a qualitative analysis of the effects observed in Example 9 of various buffers on the amplification reaction in accordance with the present invention.

Figure 11 is a southern blot (with lane legend) obtained from Example 10 in which two buffers, DMAB and DMG, were separately employed in nucleic acid production.

Figure 12 is an agarose gel and lane legend obtained in Example 11 in which the nature of the ends of amplified product was investigated.

Figure 13 is an agarose gel obtained in Example 12 in which amplification from non-denatured template was examined.

Figure 14 is an agarose gel obtained in Example 13 in which amplification from an RNA template was examined.

Figure 15 is a southern blot of the gel obtained in Figure 14.

Figure 16 is a fluorescence spectrum illustrating the results obtained in Example 14 in which the phenomenon of "strand displacement" using ethidium-labeled oligonucleotides in accordance with the present invention was investigated.

Figure 17 is a fluorescence spectrum illustrating the results obtained in Example 15 in which a T7 promoter oligonucleotide 50 mer labeled with ethidium was employed to study its effects on *in vitro* transcription by T7 and T3 polymerases from an IBI 31 plasmid (pIBI 31-BH5-2) and from a BlueScript II plasmid construct (pBSII//HCV).

Figure 18 depicts the polylinker sequences of the IBI 31 plasmid (pIBI 31-BH5-2) and the BlueScript II plasmid construct (pBSII//HCV).

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes novel methods and constructs for production of multiple copies of specific nucleic acid sequences *in vitro* and *in vivo*

One aspect of this invention represents an *in vitro* process for the production of more than one copy of nucleic acid from specific target nucleic acid (either DNA or RNA) sequences utilizing a biological catalyst, e.g., a DNA polymerase, primer oligonucleotides complementary to sequences (primer sites) in the target nucleic acid. The production process can proceed in the presence of a large excess of other nucleic acids and does not require thermal cycling or the introduction of specific intermediate constructs such as promoters or assymetric restriction sites, etc.

More particularly, this invention provides an *in vitro* process for producing more than one copy of a specific nucleic acid, the process being independent of a requirement for the introduction of an intermediate structure for the production of any such specific nucleic acid. The *in vitro* production process comprises the steps of: (a) providing a nucleic acid sample containing or suspected of containing the sequence of the specific nucleic acid; (b) contacting the sample with a three component mixture; and (c) allowing the thus-contacted mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing more than one copy of the specific

nucleic acid. The three component mixture just alluded will generally comprise (i) nucleic acid precursors, (ii) one or more specific nucleic acid primers each of which is complementary to a distinct sequence of the specific nucleic acid, and (iii) an effective amount of a nucleic acid producing catalyst. In other aspects, the specific nucleic acid may be single-stranded or double-stranded, and may take the form of deoxyribonucleic acid, ribonucleic acid, a DNA.RNA hybrid or a polymer capable of acting as a template for a nucleic acid polymerizing catalyst.

In addition, the specific nucleic acid can be in solution in which case the above-described *in vitro* process may further comprise the step of treating the specific nucleic acid with a blunt-end promoting restriction enzyme. Further, isolation or purification procedures can be employed to enrich the specific nucleic acid. Such procedures are well-known in the art, and may be carried out on the specific nucleic acid prior to the contacting step (b) or the reacting step (c). One means of isolation or purification of a nucleic acid involves its immobilization, for example, by sandwich hybridization (Ranki et al., 1983), or sandwich capture. Particularly significant in the latter methodology is the disclosure of Engelhardt and Rabbani, U.S. Patent Application Serial No. 07/968,706, filed on October 30, 1992, entitled "Capture Sandwich Hybridization Method and Composition," now allowed, that was published as European Patent Application Publication No. 0 159 719 A2 on October 30, 1985. The contents of the foregoing U.S. patent application is incorporated herein by reference.

The target nucleic can be be present in a variety of sources. For purposes of disease diagnosis these would include blood, pus, feces, urine, sputum, synovial fluid, cerebral spinal fluid, cells, tissues, and other sources. The production process can be performed on target nucleic that is present in samples which are free of interfering substances, or the production process can be performed on target nucleic acid separated from the sample. The nucleic acid can be in solution or bound to a solid support. While the replication process can be carried out in the presence of nonrelevant nucleic acids, certain applications may require prior separation of the target sequences. Methods such as sandwich hybridization or sandwich capture referenced above can then be applied to immobilize target sequences. In such instances where sandwich hybridization or sandwich capture is carried out, the above-described *in vitro* process may further comprise the step of releasing the captured nucleic acid, e.g., by means of a restriction enzyme.

As described above, the target sequence need not be limited to a double-stranded DNA molecule. Target molecules could also be single stranded DNA or RNA. For example, replication of a single-stranded target DNA could proceed using primers complementary to both the single-stranded DNA target and to the produced complementary sequence. Following the initial synthesis of the complementary sequence DNA, production from this strand would begin. RNA can serve as the template using a DNA polymerase I, e.g., Klenow, which can

reverse transcribe under conditions that have been described (Karkas, J.D. et al., Proc Nat Acad Sci U.S.A. 69:398-402 (1972)).

In case the target nucleic acid is double stranded, a restriction digest or sonication, partial endonuclease treatment or denaturation could be employed for the preparation of the target nucleic acid before the onset of amplification.

An aspect of this invention concerns its use in determining whether a specific target nucleic acid was derived from a living or a deceased organism. To make such a determination, one could in parallel amplify and detect the presence of a specific target DNA or a specific target RNA associated with the genomic makeup of the organism; and thereafter amplify and detect the presence of a specific RNA target associated to the biological function (living function) of the organism which does not survive if the organism is deceased.

The nucleic acid precursors contemplated for use in the present invention are by and large well-known to those skilled in the art. Such precursors may take the form of nucleoside triphosphates and nucleoside triphosphate analogs, or even combinations thereof. More particularly, such nucleoside triphosphates are selected from deoxyadenosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, deoxythymidine 5'-triphosphate, deoxycytidine 5'-triphosphate, adenosine 5'-triphosphate, guanosine 5'-triphosphate, uridine 5'-triphosphate and cytidine 5'-triphosphate, or a combination of any of

the foregoing. Such nucleoside triphosphates are widely available commercially, or they may be synthesized by techniques or equipment using commercially available precursors.

In the case where the nucleic acid precursors comprise nucleoside triphosphate analogs, these are also widely available from a number of commercial sources, or they may be manufactured using known techniques. Such nucleoside triphosphate analogs can be in the form of naturally occurring or synthetic analogs, or both.

It should not go unrecognized or even unappreciated that the foregoing nucleoside triphosphate and nucleoside triphosphate analogs can be unmodified or modified, the latter involving modifications to the sugar, phosphate or base moieties. For examples of such modifications, see Ward et al., U.S. Patent No. 4,711,955; Engelhardt et al., U.S. Patent No. 5,241,060; Stavrianopoulos, U.S. Patent No. 4,707,440; and Wetmur, Quartin and Engelhardt, U.S. Patent Application Serial No. 07/499,938, filed on March 26, 1990, the latter having been disclosed in European Patent Application Serial No. 0 450 370 A1, published on October 9, 1991. The contents of the foregoing U.S. patents and patent application are incorporated by their entirety into the present application.

The primers, one or more, described herein bind to specific sequences on the target nucleic acids and initiate the polymerizing reaction. While oligo deoxynucleotide primers may be preferred, polydeoxynucleotide as well as oligo and polyribonucleotide or

nucleotide copolymer primers can be used (Kornberg, A. and T. A. Baker, second edition, 1992, W.H. Freeman and Co. New York, Karkas, J.D., PNAS 69:2288-2291 (1972); and Karkas, J.D. et al., Proc. Natl. Acad. Sci. U.S.A. 69:398-402 (1972)). Thus, the specific nucleic acid primers may be selected from deoxyribonucleic acid, ribonucleic acid, a DNA.RNA copolymer, or a polymer capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization. Under conditions where the primer is an oligoribonucleotide or copolymer, the primer can be removed from its cognate binding site using specific enzymatic digestion (e.g., RNase H, restriction enzymes and other suitable nucleases) such that another primer can bind and initiate synthesis. This can be used as a system for the multiple initiation of the synthesis of polynucleotide or oligonucleotide product.

Modifications, including chemical modifications, in the composition of the primers would provide for several novel variations of the invention. See, for example, U.S. Patent Nos. 4,711,955; 5,241,060; 4,707,440; and U.S. Patent Application Serial No. 07/499,938, *supra*. For example, substitution of the 3' hydroxyl group of the primer by an isoteric configuration of heteroatoms, e.g., a primary amine or a thiol group, would produce chemically cleavable linkers. In the case of thiol excess of another thiol in the reaction mixture will cleave the phosphorothioate linkers which is formed after the initiation of polymerization, thus allowing the DNA polymerase to reinitiate polymerization with the same primer. Thus, in this variation

repeated syntheses can begin from a modified, hybridized primer providing a significant increase in the synthesis of DNA.

In another aspect of the invention, the specific nucleic acid primers are not substantially complementary to one another, having for example, no more than five complementary base-pairs in the sequences therein.

In another variation, the primer could contain some noncomplementary sequences to the target, whereupon hybridization would form at least one loop or bubble which could be used as a substrate for a specific endonuclease such that the primer could be removed from the target by enzymatic digestion thus allowing reinitiation. Furthermore, the primer could contain additional sequences noncomplementary to the target nucleic acid. Thus, the specific nucleic acid primers may comprise at least one non-complementary nucleotide or nucleotide analog base, or at least one sequence thereof. The range of non-complementarity may range in some cases from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs, and in other cases, from about 5 to about 20 nucleotides. Such noncomplementary base sequence or sequences can be linked by other than a phosphodiester bond.

As used herein, the term "nucleic acid producing catalyst" is intended to cover any agent, biological, physical or chemical in nature, that is capable of adding nucleotides (e.g., nucleoside triphosphates,

nucleoside triphosphate analogs, etc.) to the hydroxyl group on the terminal end of a specific primer (DNA or RNA) using a pre-existing strand as a template. A number of biological enzymes are known in the art which are useful as polymerizing agents. These include, but are not limited to E. coli DNA polymerase I, Klenow polymerase (a large proteolytic fragment of E. coli DNA polymerase I), bacteriophage T7 RNA polymerase, and polymerases derived from thermophilic bacteria, such as Thermus aquaticus. The latter polymerase are known for their high temperature insensitivity, and include, for example, the *Taq* DNA polymerase I. A thermostable *Taq* DNA polymerase is disclosed in Gelfand et al., U.S. Patent No. 4,889,818. Preferred as a polymerizing agent in the present invention is the *Taq* DNA Polymerase I. Many if not all of the foregoing examples of polymerizing agents are available commercially from a number of sources, e.g., Boehringer-Mannheim (Indianapolis, IND). Particularly suitable as nucleic acid producing catalysts are DNA polymerase and reverse transcriptase, or both. As used herein, "the effective amount" of the nucleic acid producing catalyst is an art-recognized term reflecting that amount of the catalyst which will allow for polymerization to occur in accordance with the present invention.

Since the rate and extent of hybridization of the primers is dependent upon the standard conditions of hybridization (Wetmur, J.G. and Davidson, N. J., Mol. Biol. 31:349 (1968)), the concentration and nucleotide sequence complexity of the total primers added to the reaction mixture will directly affect the rate at which they hybridize

and accordingly the extent to which they will initiate nucleic acid synthesis. In addition, if the reaction is run under conditions where the guanosine triphosphate is replaced by inosine triphosphate or other modified nucleoside triphosphates such that the presence of this modified nucleotide in the product nucleic acid would lower the melting temperature of the product:template double helix, then at any given temperature of the reaction the extent of breathing of the double helix will be increased and the extent of binding of the primers to the target strand will be enhanced.

Furthermore, primers could displace the strands at the ends of the double stranded target and hybridize with one of the two strands and, this displacement hybridization reaction (or D loop formation reaction) is favored by adding more than one primer molecule. In general, as the total amount of the sequence complexity of the primers complementary to the target nucleic acid is increased a greater nucleic acid production is obtained (see Example 3 below).

Modification of the primers could either increase or decrease the binding of primer to the target at a given pH, temperature and ionic strength, in other words, at isostatic conditions of pH, temperature and ionic strength, e.g., ionic salt. Other primer modifications can be employed which would facilitate polymerization from the primer sites, even when the initiation site is within a double helix. For example, once an oligo primer is introduced into a target double stranded nucleic acid molecule, if such an oligo primer is modified with ethidium or any

moiety that increases the melting temperature of the double stranded structure formed by the oligo and a target nucleic acid, it forms a relatively more stable single stranded structure because of the nucleotide modifications. This produces a primer initiation site. In fact, the nucleic acid precursors or the specific primers (or both) can be modified by at least one intercalating agent, such as ethidium, in which case it may be useful to carry out an additional step (d) of detecting any product produced in step (c), as set forth above. In such a step where desirable, detection can be carried out by means of incorporating into the product a labeled primer, a labeled precursor, or a combination thereof.

Another additional aspect of the *in vitro* process, above-described, is the inclusion of a further step of regenerating one or more specific nucleic acid primers, as described elsewhere in this disclosure, including immediately below.

As described in the summary of this invention, an *in vitro* process for multiple nucleic acid production is provided in which the products are substantially free of any primer-coded sequences. In such process, the removing step (d) is carried out by digestion with an enzyme, e.g., ribonuclease H. In one aspect of this invention, the nucleic acid precursors are modified or unmodified in the instance where one or more specific polynucleotide primers are used, the primers comprising at least one ribonucleic acid segment and wherein each primer is substantially complementary to a distinct sequence of the specific

nucleic acid. Thus, the specific polynucleotide primers may further comprise deoxyribonucleic acid. In another feature of this particular *in vitro* process, the specific polynucleotide primers contain a 3'-hydroxyl group or an isoteric configuration of heteroatoms, e.g., nitrogen, sulfur, or both. In addition, the polynucleotide primers in this instance may further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs.

In yet a further *in vitro* process for producing more than one copy of a specific nucleic acid is provided (as described in the summary), the products being substantially free of any primer-coded sequences. In this instance, unmodified nucleic acid precursors are reacted in a mixture with one or more chemically-modified primers each of which is substantially complementary to a distinct sequence of the specific nucleic acid. An effective amount of a nucleic acid producing catalyst is also provided in the mixture. As in the case of the last-described *in vitro* process, the removing step (d) may be carried out by digestion with an enzyme, e.g., ribonuclease H. The specific chemically modified primers are selected, for example, from ribonucleic acid, deoxyribonucleic acid, a DNA.RNA copolymer, and a polymer capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization, or a combination of any of the foregoing. The specific chemically modified primers may contain a 3'-hydroxyl group or an isosteric configuration of heteroatoms, N, S, or both, as described above in other *in vitro* processes. Further, the specific chemically modified primers can be selected from nucleoside

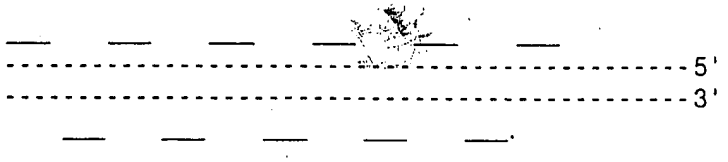
triphosphates and nucleoside triphosphate analogs, or a combination thereof, wherein at least one of said nucleoside triphosphates or analogs is modified on the sugar, phosphate or base. Also as in other *in vitro* processes, the specific chemically modified primers may further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs.

In still yet another of the *in vitro* processes for multiple nucleic acid production, described previously in the summary of this invention, unmodified nucleic acid precursors are provided in the mixture and reacting step (c), together with one or more specific unmodified primers comprising at least one segment, each of which primer comprises at least one non-complementary sequence to a distinct sequence of the specific nucleic acid, such that upon hybridization to the specific nucleic acid at least one loop structure is formed. As in the other instances, digestion with an enzyme, e.g., ribonuclease H, may be employed in the removing step (d). In one feature of this process, specific unmodified primers are selected from ribonucleic acid, deoxyribonucleic acid, a DNA.RNA copolymer, and a polymer capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization, or a combination of any of the foregoing. Further, the specific unmodified primers may further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs, in accordance with the present invention.

The rate of hybridization of the primer to target nucleic acids and, in particular, to target double stranded nucleic acids can be facilitated by binding of the primer with various proteins, e.g., rec A proteins. For example, if the primer is modified with an intercalating agent, e.g., ethidium (or any moiety that increases the melting temperature of the double stranded structure), the addition of this primer to or with a protein such as rec A, either free or bound, would facilitate the introduction of the primer into the double stranded target. (Kornberg and Baker, *supra*, pages 797-800). This could produce a suitable primer initiation site.

The arrangement of primer binding sites on the template nucleic acid can be varied as desired. For example, the distance between successive primer binding sites on one strand can also be varied as desired. Also specific primers can be employed that initiate synthesis upstream of the sequence sought to be copied. Under this scenario, multiple copies of nucleic acid are made without successive denaturation or use of other enzymes or the introduction of intermediate structures for their production.

When primer sites on double stranded DNA are arranged as shown, specific DNA production is increased.



When the target sequences are substantially covered by their complementary primers, a further increase in the production of multiple copies of nucleic acid is favored due to the increase in initiation points and destabilization of the double stranded template molecule.

Finally, if an oligo is modified such that it will form a stable hybrid, even in the presence of the complementary nucleic acid strand, then the modified oligo can act as a 'helper' oligo. 'Helper' oligo in this context is defined as a oligo that does not necessarily act as a primer but will accelerate the binding and priming activity of other oligos in the vicinity to the binding site of the 'helper' oligo. Vicinity is here being defined as the location of a nucleotide sequence or the complementary nucleotide sequence close enough to the binding site of the 'helper' oligo to have the rate or extent of hybridization of the primer affected by the binding of the 'helper' oligo. The 'helper' oligo can be modified such that it does not initiate polymerization as for example through the use of a dideoxy 3' terminal nucleotide or other nucleotide with blocked 3' ends. The 'helper' oligo can also be modified in such a manner that the double helix formed by the 'helper' oligo and the target nucleic acid strand or the 'helper' and the complementary strand to the target strand is more stable or has a higher melting temperature than the equivalent double helix of unmodified 'helper' oligo and the target or the strand complementary to the target strand. Such modifications can include halogenation of certain bases, ethenyl

pyrimidines (C:C triple bonds, propyne amine derivatives; the addition of ethidium or other intercalating molecules (see Stavrianopoulos and Rabbani, U.S. Patent Application Serial No. 07/956,566, filed on October 5, 1992, the contents of which are incorporated herein by reference and which were disclosed in European Patent Application Publication No. 0 231 495 A2, published on August 12, 1987); the supplementation of the oligo with certain proteins that stabilize the double helix and any other treatment or procedure or the addition of any other adduct that serves to stabilize the portion of the double helix with the 'helper' bound or to increase the melting temperature of portion of the double helix with the 'helper' bound.

In vivo Synthesis of Nucleic Acid

This invention describes a casette or nucleic acid construct into which any nucleic acid sequence can be inserted and which can be used as a template for the production of more than one copy of the specific sequence. This cassette is a nucleic acid construct containing a sequence of interest, which within or present within, the cell produces nucleic acid product which is independent or only partially dependent on the host system. The cassette or nucleic acid construct may be characterized as a promoter-independent non-naturally occurring, and in one embodiment comprises double-stranded and single-stranded nucleic acid regions. This construct contains a region in which a portion of the opposite strands are not substantially complementary,

e.g., a bubble (even comprising at least one polyT sequence), or loop, or the construct comprises at least one single-stranded region. The construct is composed of naturally occurring nucleotides or chemically modified nucleotides or a synthetic polymer in part or a combination thereof. These structures are designed to provide binding of polymerizing enzymes or primers and the modifications provide for nuclease resistance or facilitate uptake by the target cell.

Referring to the constructs (A-F) depicted in Figure 3, the single stranded regions described in the constructs will contain coding sequences for nucleic acid primers present in the cell to facilitate initiation points of DNA polymerase in said cell. In the case of RNA polymerase, these constructs constitute promotor independent binding and initiation of RNA polymerase reaction. These constructs can be used *in vitro* and *in vivo* for production of nucleic acids. The position of the single stranded region adjacent to the double stranded specific sequence would provide a specific and consistent transcription of these specific sequences, both *in vitro* and *in vivo* independent of promotor. The replication (DNA) or transcription (RNA) products of these constructs can be single stranded nucleic acid which could have a sense or antisense function or could be double stranded nucleic acid.

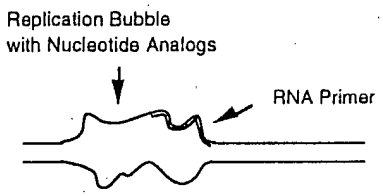
In Figure 13(A), a large bubble is located in the construct. In Figure 13(B), the two strands are noncomplementary at their ends, and thus do not form a bubble. In Figure 13(C), a double bubble is formed due to noncomplementarity at both ends. In Figure 13(D), a single-

stranded region is shown in the middle of the construct leading to a partially single-stranded region (and no bubble formation). Figure 13(E) depicts a bubble at one end of the construct (compare with the two bubbles in the construct shown in Figure 13(C)). In Figure 13(F), a single bubble in the middle of the construct is shown. It should be readily appreciated by those skilled in this art that the above-depicted embodiments are representative embodiments not intended to be limiting, particularly in light of the present disclosure.

In vivo these constructs, with a specific primer present in the cell can initiate nucleic acid synthesis. When these primers are RNA, after initiation of nucleic acid synthesis, they can be removed by the action of ribonuclease H, thus vacating the primer binding sequence and allowing other primer molecules to bind and reinitiate synthesis. The cellular nucleic acid synthesizing enzymes can use these constructs to produce copies of a specific nucleic acid from the construct. Shown in Figure 14 (A-F) are corresponding illustrations of the constructs in Figure 13 (A-F), except that the production arrows (points and directions) are indicated.

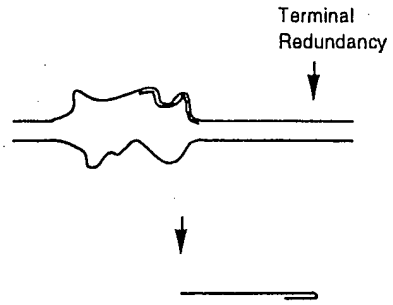
These constructs could contain more than one specific nucleic acid sequence which in turn could produce more than one copy of each specific nucleic acid sequence. If two independent nucleic acid products are complementary, then they could hybridize and form muliple copies of a new double stranded construct that could have the properties of the novel construct. Furthermore they could contain

promotor sites such as the host promotor therefore serving as an independent nucleic acid production source (the progeny).



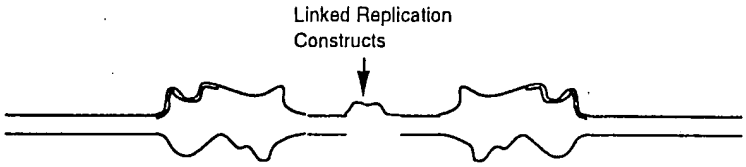
Primer-Dependent DNA Production Using Nucleic Acid Construct

The replication of this structure could result in the production of one strand of DNA product. Several alternative events may occur allowing for the formation of a second complementary strand. For example, a terminal loop could be inserted at the end of the construct such that the single stranded product will code for the synthesis of the complementary strand using the repair enzyme. Constructs can be made that produce single stranded DNA product that has a hairpin loop and therefore, can be used to form a double-stranded product. Alternatively, constructs can be formed that produce nucleic acid in both polarity.



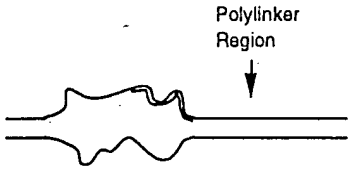
Hairpin Product

An alternative approach to the production of double stranded product is to covalently link two constructs that make complementary DNA strands.



Linked Complementary Production Constructs

The construct can be made to contain a poly linker region into which any sequence can be cloned. The result will be a transient accumulation of expressing genes within the cell to deliver sense, antisense or protein or any other gene product into the target cell.



Cloning Site in Production Constructs

Other processes within the invention herein described apply to the production of more than one copy of functional genes or antisense DNA or RNA in target cells.

Production of Primers

Primers can be produced by several methods. Single-stranded oligonucleotides in the range from between from about 5 to about 100 bases long, and preferably between from about 10 to about 40, and more preferably, between from about 8 to about 20 nucleotides. These ranges may further vary with optimally between from about 13 to about 30 for bacterial nucleic acid, and optimally between from about 17 to about 35 for eukaryote nucleotides would appear to be appropriate for most applications although it may be desirable in some or numerous instances to vary the length of the primers. Oligonucleotide primers can be most conveniently produced by automated chemical methods. In this way modified bases can be introduced. Manual methods can be used and may in some cases be used in combination with automated methods. All of these methods and automation are known and available in the art.

In addition nucleic acid primers can be produced readily by the action of T7 RNA polymerase, T3 polymerase, SP6 polymerase or any appropriate DNA or RNA polymerase on DNA templates or RNA templates containing the primer sequences extended from the corresponding RNA polymerase promoter sites or other nucleic acid synthesis start signals.

Detection of Products

DNA produced by the invention described herein can be detected by a variety of hybridization methods using homogeneous or non-homogeneous assays. DNA produced in tissues or cells, i.e., *in situ*, can be detected by any of the practiced methods for *in situ* hybridization. These include, but are not limited to, hybridization of the produced DNA with a nucleic acid probe labeled with a suitable chemical moiety, such as biotin. Probes used for the detection of produced DNA can be labeled with a variety of chemical moieties other than biotin. These include but are not limited to fluorescein, dinitrophenol, ethidium (see, for example, the disclosures of U.S. Patent Nos. 4,711,955; 5,241,060; and 4,707,440, *supra*).

The hybridized, labeled nucleic acid probe can be detected by a variety of means. These include but are not limited to reaction with complexes composed of biotin binding proteins, such as avidin or streptavidin, and color generating enzymes, such as horseradish

peroxidase or alkaline phosphatase, which, in the presence of appropriate substrates and chromogens, yield colored products.

In accordance with this invention, DNA production from target sequences generally requires nucleic acid precursors, e.g., adenosine triphosphate, guanosine triphosphate, thymidine triphosphate and cytosine triphosphate, present in sufficient quantity and concentration in the reaction mixture. In other applications it may be advantageous to substitute one or more of the natural precursors with modified nucleotides. For example, when the invention described herein is being applied to the detection of specific nucleic acid sequences, immobilization of the produced DNA may be desirable. In such an instance, substitution of one or more of the natural nucleotide triphosphate precursors with a modified nucleotide, e.g., biotinylated deoxyuridine triphosphate, in place of thymidine triphosphate, would yield biotin-labeled DNA. The produced DNA could be separated by its affinity for a biotin binding protein, such as avidin, streptavidin or an anti-biotin antibody. A variety of nucleoside triphosphate precursors (U.S. Patent Nos. 4,711,955; 5,241,060; and 4,707,440, *supra*) labeled with chemical moieties which include, but are not limited to, dinitrophenol and fluorescein, and which can be bound by corresponding antibodies or by other binding proteins can be used in this manner. In other aspects of the invention, the produced DNA can be isotopically labeled by the inclusion of isotopically labeled deoxynucleotide precursors in the reaction mixture.

Labeled DNA, produced by the invention described herein, can function as probe nucleic acid to be used to detect specific target nucleic acid sequences.

In certain detection formats the primers may be removed from the reaction mixture by capturing the product through direct capture (Brakel et al., U.S. Patent Application Serial No. 07/998,660, filed on December 23, 1992, the contents of which have been disclosed in European Patent Application 0 435 150 A2, published on July 3, 1991; and the contents of which are also incorporated by reference herein), or sandwich capture. (Engelhardt and Rabbani, allowed U.S. Patent Application Serial No. 07/968,706, *supra*), or by modifying the primers at the 3' end with biotin or imminobiotin without an arm or a very short arm such that the avidin will recognize only the unincorporated primers (single stranded form) but not the incorporated due to the double stranded form and the short length of the arm. Additionally, the primer may be labeled with ethidium or other intercalating moiety. In this condition, the ethidium or other intercalating moiety may be inactivated (Stavrianopoulos, U.S. Patent Application Serial No. 07/633,730, filed on December 24, 1990, published as European Patent Application Publication No. 0 492 570 A1 on July 1, 1992; the contents of which are incorporated by reference) in the unhybridized oligo and not in the hybridized oligo:target.

Another aspect of this invention herein described is to provide for a conjugate of a nucleic acid polymerizing enzyme (RNA

polymerase) with a nucleic acid construct said nucleic acid construct contains an initiation site such as a promotor site for the corresponding RNA polymerase which is capable of producing nucleic acid both *in vivo* and *in vitro*. The enzyme could be linked directly to the nucleic acid or through a linkage group substantially not interfering with its function or the enzyme could be linked to the nucleic acid indirectly by a nucleic acid bridge or haptene receptor where the enzyme is biotinylated and the nucleic acid construct contains an avidin or *vice versa* or when the nucleic acid construct contains sequences for binding proteins such as a repressor and an enzyme linked to said nucleic acid binding protein (U.S. Patent No. 5,241,060, *supra*, and Pergolizzi, Stavrianopoulos, Rabbani, Engelhardt, Kline and Olsiewski, U.S. Patent Application Serial No. 08/032,769, filed on March 16, 1993, published as European Patent Application Publication No. 0 128 332 A1 on December 19, 1984, the latter having been "allowed" by the European Patent Office, and further incorporated by reference herein).

Further in regard to the just-described conjugate of the present invention, the protein in one aspect comprises an RNA polymerase or a subunit thereof and the nucleic acid construct contains the corresponding RNA polymerase promoter. The RNA polymerase can be selected from T7, T3 and SP6, or a combination of any of the foregoing. In another embodiment, the protein in the conjugate comprises DNA polymerase or reverse transcriptase and the nucleic acid construct contains at least one sequence complementary to an RNA molecule. The

construct can take the form of double-stranded, single-stranded, or even partially single-stranded. Further, the nucleic acid construct in the conjugate may comprise at least one chemically modified nucleotide or nucleotide analog. The linkages of the protein to the construct are described in the preceding paragraph. The nucleic acid produced by or from this conjugate comprises deoxyribonucleic acid, ribonucleic acid, or combinations thereof, or it may be antisense or sense, or both.

As described in the summary of the invention, the above-described conjugate may be utilized in an *in vivo* process for producing a specific nucleic acid. In other aspects of this *in vivo* process, the construct is further characterized as comprising (independently) at least one promoter, at least one complementary sequence to a primer present in the cell, or codes for the protein in the conjugate, or for a protein other than the protein in the conjugate. The other protein may comprise a nucleic acid polymerase. In the instant where the polymerase comprises an RNA polymerase, the nucleic acid construct may comprise a promoter for the RNA polymerase. Further, the polymerase can be a DNA polymerase or reverse transcriptase.

(a) Direct Attachment of a Polymerase to the Construct

For example, if a construct containing a RNA polymerase linked directly or indirectly to a DNA construct or cassette is introduced into

a cell, the RNA polymerase will transcribe the nucleic acid in the construct and is completely independent of any host RNA polymerases. Each molecule introduced into a cell will produce multiple copies of a segment of the construct. In the first iteration, the attached polymerase can produce the RNA for the target sequence itself. (See Figure 3 (A)). Alternatively, the promotor, specific for the attached polymerase, may be linked to two separate sequences, namely the polymerase gene and the target gene. See Figure 3 (B). In this instance, the amount of polymerase initiating at the promotor site will increase as the polymerase gene is transcribed and translated. Finally, the coding sequence transcribed by the T_7 promotor (or any specific first promotor) may produce any RNA polymerase (including T_7 polymerase or polymerase III or others), and this polymerase may transcribe off of another or second promotor (or off of a different strength T_7 or other first promotor) to produce the transcript of the target sequence. (See Figure 3 (C)).

Referring to the constructs or cassettes shown in Figure 4 (A-C), these can be derived by using standard recombinant DNA techniques. The appropriate piece of DNA can be isolated and covalently attached to the RNA polymerase under conditions whereby the RNA polymerase functions after being covalently attached to a solid matrix (Cook, P.R and Grove, F. Nuc. Acids Res., 20:3591-3598 (1992)). Methods of modifying the ends of DNA molecules for attachment of chemical moieties are well known (see, for example, U.S. Patent Application Serial No. 08/032,769, *supra*). The transcribed product can act per se

as sense or antisense RNA or it can be translated into protein. The enzyme and/or nucleic acid constructs could be modified to facilitate transport and/or achieve resistance to degrading enzymes (U.S. Patent No. 5,241,060, *supra*).

(b) *In vivo* Amplification of Transcription

Constructs can be made that are dependent upon transcription or replication using a host polymerase. When such a construct contains a double promotor, the second promotor can be different than the first promotor or it can be a stronger or weaker version of the first promotor. Vectors can be chosen such that the constructs can either integrate into the chromosome, replicate autosomally or be replication-deficient and function only for transient expression. They can function in the nucleus or the cytoplasm if the target cell is eukaryotic. The figure below depicts various constructs or cassettes and is not limiting as to the possible variations contemplated by the present invention.

Referring to Figure 4 (A), the nucleic acid construct or cassette depicted in this figure contains a promotor that codes for the production of a polymerase that is not endogenous to the target cell. For example, an SV40 or RNA polymerase III promotor that codes for a T_7 RNA polymerase. Transcription and translation of these transcripts by cellular machinery results in the production of active T_7 RNA

polymerase which will utilize the T_7 promotor to transcribe the target sequence (Fuerst, T.R. et al., Proc Nat Acad Sci USA 83:8122 (1986)) have shown high levels of transient expression using a dual construct system with the T_7 RNA polymerase gene on one construct and the target gene behind the T_7 promotor on the other construct. The simplest iteration of this construct is that the genes coding for the polymerase code for a polymerase that exists within the cell and therefore is not recognized by the host organism as a foreign protein and does not induce an immune response.

In Figure 4 (B), an additional autocatalytic cycle has been added whereby the extent of transcription of the target gene is enhanced by the production of T_7 RNA polymerase throughout the transient expression cycle.

In Figure 4 (C), the construct or cassette is similar to Figure 4 (B) with the additional element that there is a down regulation of the autocatalytic cascade by competition by a high efficiency promotor with a low efficiency transcriptional promotor.

Three Constructs with Promotors for Endogenous RNA Polymerase

As described in the summary, the present invention further provides a construct comprising a host promoter located on the construct such that the host transcribes a sequence in the construct

coding for a different RNA polymerase which after translation is capable of recognizing its cognate promoter and transcribing from a DNA sequence of interest in the construct with the cognate promoter oriented such that it does not promote transcription from the construct of the different RNA polymerase. In one feature of this construct, the host promoter comprises a prokaryotic promoter, e.g., RNA polymerase, or eukaryotic promoter, e.g., Pol I, Pol II, Pol III, or combinations thereof, such prokaryotic or eukaryotic promoter being located upstream from the host promoter. The second RNA polymerase may be selected from various members, including T7, T3 and SP6, or combinations thereof. The DNA sequence of interest may comprise sense or antisense, or both, or it may comprise DNA or RNA, or still yet, it may encode a protein. The construct may further comprise at least one chemically modified nucleotide.

Additionally, promotors that will be read by polymerases within the target cell can be linked to the production of additional polymerase specific for that promotor or other promotors. The polymerases can be for example, T7 polymerase, RNA polymerase III, or any other polymerase. A second promotor keyed sequence can be in the construct such that a second polynucleotide can be synthesized from the construct. It can code for the production of antisense or sense RNA or DNA molecules. These constructs or cassettes can be created using standard recombinant DNA techniques.

The property and structure of all nucleic acid constructs provided in accordance with this invention is applicable to each other in combination, *in toto* or in part. That is to say, in the conjugate comprising a protein and a nucleic acid construct, the construct could include, for example, chemical modification and bubble structure or single-stranded regions for primer binding sites or RNA initiation sites. Other variations would be recognized by those skilled in the art in light of the detailed description of this invention.

The examples which follow are set forth to illustrate various aspects of the present invention but are not intended to limit in any way the scope as more particularly set forth in the claims below.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

EXAMPLES

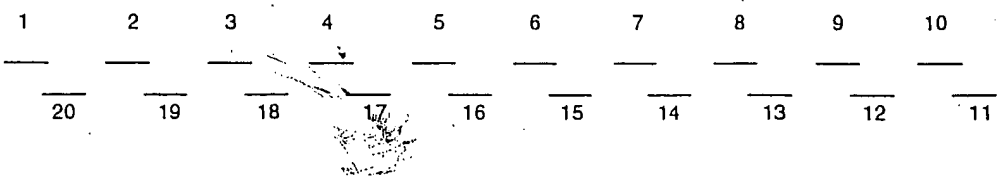
Example 1

Primers

A set of twenty single stranded oligonucleotide primers, fifteen nucleotides long, were chemically synthesized.

The first set of 10 primers was complementary to one strand of M13mp18 replicative form (RF) starting at base 650 and extending to base 341. An interval of 15 nucleotides separated successive primers. The second set of 10 primers contained sequences identical to the single-stranded M13mp18 phage genome starting at base 351 and extending to base 635, again with 15 nucleotide gaps separating successive primers. There is a complementarity of 5 bases between opposing primers, but at an ionic concentration of 0.08M NaCl and 45°C these primers will not hybridize to each other. The sequences of the primers are shown in Figure 6.

ARRANGEMENT OF OLIGONUCLEOTIDE PRIMERS IN AMPLIFICATION REACTION



Primer 1 begins at base 650 and primer 11 begins at base 351.

Example 2

Amplification Target

The target of amplification was the M13mp18 RF. This was digested with either *Taq*1 or a combination of *Bam*H1 and *Eco*R1. *Eco*R1 and *Bam*H1 cut at sites close to each other and digestion with either enzyme alone would transform the circular RF molecule into a linear DNA molecule. The *Taq*1 enzyme digests M13mp18 RF yielding 12 fragments. The sequence to be amplified (nucleotides 351 to 650) was flanked in the *Bam*H1/*Eco*R1 digested RF by two regions, 1,371 bases and 5,601 bases, and *Taq*1-digested M13mp18 RF was flanked by regions of 15 and 477 nucleotides (see Figure 7).

In amplification experiments, the restriction digests were used without any further purification. For amplification, a control of irrelevant DNA (calf thymus) was employed.

The precursors were added in 50µl aliquots. One 10µl aliquot of the precursors was mixed with 90µl H_2O and loaded on a glass fiber filter, dried and counted. The counts were multiplied by 5 and divided by 160 (nmoles in the incubation mix). Specific activity is the cpm/nmoles of nucleotides.

Amplification is measured as follows. The total counts are determined and this number is divided by the specific activity of the precursors to determine the number of nanomoles of incorporation. The target (in n grams)

is divided by 330 (average molecular weight of nucleotide) to determine the nanomoles of input target phosphate. The amplification is then calculated by dividing the nanomoles of product by the nanomoles of input target.

Example 3

The Effect of Primer Concentration on the Amplification of Target DNA.

An incubation mixture of 130 µl contained the following reaction components: 40 mM sodium phosphate, pH 7.5, 400 µM each of the four deoxynucleotide triphosphates, 5 mM dithiothreitol, 40 ng of Taq1-digested M13mp18 RF (containing 3.5 ng of the *Taq*1 fragment to be amplified), and all 20 primers (at 0.04 OD/ml, 0.4 OD/ml or 0.8 OD/ml) and 15 units of Klenow fragment of DNA polymerase. The mixture was left at room temperature for 20 minutes in order to allow the enzyme to cover all of the initiation sites on the template. The polymerization was then initiated by the addition of Mg^{++}, 7 mM final concentration, and the tubes were placed in a 45°C bath. After 1 hour an additional 15 units of the enzyme were added, and the incubation was continued for another hour. The reaction was stopped with 100 µmoles of EDTA, 100 µg sonicated calf thymus DNA were added, and the nucleic acids were precipitated with 1.0 ml of cold 10% TCA for 60 minutes at 0°C. The mixture was filtered through a glass fiber filter, the filter was washed 3 times with cold 5% TCA, then twice with ethanol, dried and counted in a Beckman liquid scintillation counter.

The specific activity of the nucleotide precursors was 9,687 cpm/nmole. The tagged *Taq1 DNA* fragment contained 0.0106 nmoles of nucleotides.

Primer Concentration	Incorporation (cpm)	Incorporation (nmoles nucleotide)	Amplification
0.04 OD/ml	32,482	3.35	316
0.4OD/ml	366,260	37.8	3566
0.8 OD/ml	512,260	52.88	4988

Example 4

The Random Priming Activity of the Primers on Calf Thymus DNA.

To test for the effect of the primers on the background, an assay was performed, as described in the preceding example (Example 3 above), in which background was determined with and without primers as well as with and without melting of the calf thymus DNA.

The amplification conditions were the same as in Example 1 except that only 5 ug (15.0 nmoles) calf thymus DNA were used as a target. The DNA employed was double stranded or heated at 100°C for 10 minutes in the presence or absence of primers (0.4 OD/ml each) before being chilled on ice.

Double Stranded DNA	Melted DNA	Primers	Incorporation cpm	Incorporation umoles	Amplification
+			239,100	24.68	1.64
+		+	276,540	28.54	1.90
	+	+	556,560	57.45	3.83
	+		28,432	2.93	0.19

This experiment suggests that the random priming activity of the primers is not substantial, that incorporation on double stranded DNA is due to the nicks on the DNA molecules, and that melting abolishes to a large extent the priming positions on the irrelevant DNA.

Example 5

Amplificiation of the M13 Fragment in the presence of a large Excess (1500-Fold) of Irrelevant DNA

The amplification conditions were the same as in Example 1. Primers (0.4 OD/ml), 5 ug calf thymus DNA and 40 ng M13mp18 DNA containing 3.5 ng of fragment were employed in this example.

Calf Thymus DNA	M13mp18 DNA	Incorporation cpm	Incorporation nmoles	Amplification
+		575,440	59.4	3.96x
	+	338,900	35.0	3,300x
+	+	713,440	73.6	

The experimental results above show that the target can be amplified in the presence of large amounts of irrelevant DNA. The net amplification was 1,343 even though in this case the target DNA inhibits the amplification of the irrelevant DNA by competing for initiation points. It is possible that the amplification was even larger.

These experimental results were also analyzed by running the samples on a 2% agarose gel. In Figure 8 it can be seen that the calf thymus template (lane 3) only gives high molecular weight DNA bands composed of a mixture of input DNA as well as DNA synthesized by random priming (as seen in the incorporation figures in the Table above given for this example). On the other hand, it can be seen that the mp18 template (lane 2) gives a distinct pattern of lower molecular weight bands, and in lane 1, similar bands are observed when the mp18 template was mixed with 1500 times as much calf thymus DNA demonstrating that the foreign DNA did not significantly affect the amplificiation of DNA from the mp18 template.

Example 6

Amplification of Different Restriction Digests

The incubation conditions were the same as in Example 4. Forty nanograms of total M13mp18 DNA were used in each experiment with 0.4 OD/ml primers. In one case, the M13mp18 DNA was cut in only one position (using *Eco*R1) leaving the fragment to be amplified flanked by two large pieces. In the other case where the RF was treated with *Taq*1, the fragment was contained in one 639 base pair fragment. The specific activity of the precursors was 8.385 cpm/nmole.

	Incorporation cpm	Incorporation nmoles
Large Fragment	393,480	46.92
Small Fragment	262,808	31.34

These experimental results show that the enzyme does not extend polymerization very far from the region where the primers hybridize, otherwise a much larger incorporation using the large piece would have been otherwise expected because the elongation of the primers by the enzyme can extend in both directions.

Example 7

A Comparison of Synchronized and Unsynchronized Reactions

In all of the preceding experiments, the enzyme was preincubated with the target-primer mixture to allow binding of the enzyme at the 3' end of the hybridized primers on the target, followed by the addition of magnesium to initiate polymerization. The conditions were the same as in Example 1.

To assay the effect of this synchronization on the extent of the reaction, the incorporation in a synchronized reaction was compared to an unsynchronized reaction initiated by adding magnesium to the complete reaction mix before enzyme addition. The reaction conditions are described in Example 3. The specific activity was 9687 cpm/nmole.

	Incorporation cpm	Incorporation nmoles	Amplification
Synchronized	495,080	51.1	4818
Unsynchronized	416,380	42.9	4052

The results above demonstrate that synchronization of the reaction is not essential for the amplification reaction.

Example 8

The Effect of Variations of the Reaction Conditions on the Product Produced by the Procedure of Example 3

A reaction was performed according the the reaction conditions of Example 3 in which twenty primers were added to the reaction mixture as well as the *Taq 1* fragments (40 nanograms, i.e., 3.5 nanograms of insert that will hybridize with the primers) described in Example 3 with the exception that the buffer was altered. In the first lane of the gel shown in Figure 9, the reaction was performed without target DNA added. In lane 2 the reaction was performed in a phosphate buffer (0.04 M, pH 7.5). Lane 3 contains the molecular weight buffers of *Msp I* digestion of pBR322 DNA. In the fourth lane the reaction was performed in which the phosphate buffer was substituted by MOPS buffer at 0.1 M and pH 7.5 (measured 25°C). It can be seen that the reaction in the phosphate buffer produced an agglomeration of DNAs that when dissociated by heat or other double helix disrupting agents lead to an number of products of a size smaller than the agglomeration structures. The size distribution of the products in the MOPS-buffered reaction corresponds to the distances between certain of the oligo primer binding sites. The smallest is approximately 76 nucleotide pairs in size which is approximately the distance between the closest specific oligo primer binding sites.

Example 9

Effect of Various Buffers on the Amplification Reaction.

The buffer used for the amplification reaction can have significant effects upon the degree of amplification. In the following example, phosphate buffer (which was employed in Example 7) was compared with the following zwitterion buffers:

4-morpholinoethyl sulfonate (MES),
4-morpholinopropionyl sulfonate (MOPS),
N-dimethylaminobutyric acid (DMAB), and
N-dimethylglycine (DMG).

Trizma base was used to adjust MES or MOPS to pH 7.5, DMAB to pH 7.8, and DMG to pH 8.6. In the previous experiments, 4.0 ng of mp18 (containing 3.5 ng of the target fragment) was used as a template. In this experiment, the amount of template was reduced ten-fold compared to those experiments (4 ng of mp18; 350 pg of target fragment). Other changes in the experimental procedure was the omission of DTT and the use of a single addition of 10 units of Klenow polymerase. Mg^{++} and dNTP concentrations (7.5 mM and 400 µM each dNTP) were as described previously.

As before, reactions were preincubated at room temperature for 30 minutes prior to the addition of the Mg^{++}. After addition of Mg^{++}, reactions were immediately transferred to a 45°C water bath and incubated for 4

hours. The reaction was stopped by the addition of 5µl of 500 mM EDTA to give a final concentration of approximately 20 mM.

For evaluation of the extent of polymerization, an aliquot of 40 µl (out of a 120 µl incubation mix) was mixed with 50 µg of sonicated calf thymus DNA and precipitated on ice with 1 ml of 10% TCA. After one hour, the precipitate was collected on glass fiber filters, washed 3 times with 5% of cold TCA, 2 times with 95% ETOH, dried and counted in a liquid scintillation counter. The input was measured by the addition of radioactive precursor onto a filter without precipitation with TCA and counted as before. The results are given in the table below. As controls, the reactions were also carried out without the addition of any target template.

Buffer	Incorporation From Template (in cpm)	No Template Control (in cpm)	Template-Specific Incorporation (in cpm)	Net Synthesis (nanomoles)	Amplification Factor
Phosphate	4,008	2,628	1,380	0.255	240
MES	299,367	212,778	86,589	16.03	15,123
MOPS	184,500	49,521	114,979	21.28	20,075
DMAB	207,239	5,859	211,380	39.13	36,915
DMG	182,655	32,012	150,643	27.89	26,311

Compared to the no template control, the highest efficiency of amplification was obtained with the DMAB buffer. The results of this experiment demonstrated that an amplification of the target region

approaching 37,000 fold could be obtained. It should be noted that another buffer, MES, gave higher incorporation, but the no template control demonstrated that there was non-specific polymerization leading to a net amplification of only 20,000 fold. The next best buffer system was DMG where net amplification was over 26,000 fold, followed by MOPS with 20,000 fold amplification.

The results of this experiment were also analyzed qualitatively by ethanol precipitating the remaining 80 µl of the reaction mixtures, resuspending them in 80 µl of TE buffer and running 10 µl aliquots on 2% agarose gels. These results are shown in Figure 10 and agree with the results shown in the table above.

Example 10

Incorporation of radioactive precursors measures total synthesis of DNA including both specific as well as template-independent DNA synthesis. Oligos No. 1,3,5,7,9,12,14,16,18 and 20 from Example 1 were employed in a series of amplification reactions. This limited number was chosen such that there would be a region within the amplicon that does not correspond to any of the primers, allowing the use of a 30 base probe (bases 469 to 498) labeled with biotin that corresponds to this open region.

The experimental design was to use DMAB and DMG buffers. Example 9 had previously shown little or no template-independent synthesis with DMAB

and substantial template-independent synthesis with DMG. Reactions with and without Taq digested mp18 were carried out. The reaction mixtures were precipitated with ethanol, resuspended in TE buffer and aliquots were electrophoresed through a 2% agarose gel. A southern blot was made from this gel and probed with 200 ng/ml labeled oligo in 31% formamide/ 2X SSC at 25°C for 2 hours and washed 3 X with 0.1X SSC/0.1% Triton X-100 for 5 minutes each at 37°C. Membranes were developed using an alkaline phosphate detection system obtained from Enzo Biochem, Inc.

As seen in Figure 11, this set of experiments demonstrates that the product of the amplification is strongly dependent upon the specific buffer used in the reaction. The best results were obtained with DMAB buffer where essentially no incorporation (data not shown) or hybridization (Figure 11, lane 1) with the reaction mixture from the no template control sample. The template dependent synthesis with DMAB (Figure 11, lane 2) produced DNA that hybridized with the labeled probe.

The nature and origin of the non-template derived synthesis achieved with DMG buffer (Figure 11, lane 3) is still under current study.

Example 11

Determination of the Nature of the Ends of the Amplified Product

If the product strands act as the template for polymerization of nucleic acid then the products should have blunt ends. One method of assaying for the presence of blunt ends is based on the notion that these molecules will undergo blunt end ligation. Molecules with 'ragged' ends (single stranded tails on the 3' or 5' end) will not participate in the ligation reaction.

Because the amplified product is initiated using chemically synthesized primer molecules, the 5' ends will not under phosphorylation. The first step of this proof will be to phosphorylate the 5' ends of both single stranded and double stranded molecules. These 5' phosphorylated molecules will then be ligated using the T4 DNA ligase. The unamplified DNA will act as the negative control and a PCR-generated amplicon will act as the positive control.

As can be seen in the gel reported in Figure 12, T4 ligase treatment increases the molecular weight of the amplified product selectively. This is most apparant in lane 4 of Figure 12, where there is an appreciable increase in size observed as a result of the completed ligation reaction.

The positive control with the PCR-generated amplicon (primed by oligos initiating at nucleotide 381 and from nucleotide 645 of the template which predicts an amplicon of 264 nucleotides) also show a shift in position

after ligation (lane 7). Because there was not much DNA included in this reaction, the appearance of a spectrum of multimers of the amplicon was not observed, but the loss of material from the position of the unligated material (lanes 5 and 6) was noted. Some material left at the position of the untreated amplicon in lane 7 was also noted. It is possible that this material does not participate in the ligation reaction because of the addition of A to the 3' end of the amplicon which is a property of the Taq polymerase.

Example 12

Amplification from non-denatured template

To explain the high level of amplification in this system, it was assumed that some of the primers might be able to initiate DNA synthesis by inverting the ends of double-stranded DNA products synthesized during amplification. This "breathing" was demonstrated in the following experiment. The template was a blunt-ended double-stranded DNA molecule obtained from Dr. Christine L. Brakel, the blunt ends extending from bases 371 to 645 in the mp18 DNA sequence. These ends exactly match primers Nos. 1 and 12 (described in Example 1). In this experiment, no radioactive precursors were used. Analysis was performed by gel electrophoresis through 2% agarose. Reagent conditions were the same as Example 10 where DMG was used as the buffer, but only 2 primers, No. 1 and No. 2 were used and no denaturation of the starting template was performed. In Figure 13, for comparison purposes, the same amount of DNA was used as the input on the

gel (lane 1). In lane 2, no template was added. In lane 3, the complete reaction mix is shown. In lane 4, 12 times as much DNA as the template input in either lanes 1 or 3 has been included as a size marker. In both lanes 2 and 3, some non-specific synthesis can be seen, but the specific copying of the template can clearly be distinguished in lane 3. Therefore, as lane 3 indicates, newly synthesized DNA of the same size as the input was formed using non-denatured double-stranded DNA, proving that the double-stranded blunt ends can serve as initiation points for replication.

Example 13

Amplication from RNA template

Although it has been demonstrated by the present invention that DNA can be amplified, it would be useful, however, to also be able to employ RNA as a potential template. Accordingly, the double-stranded DNA molecule used in Example 12 was ligated into the Sma I site of a vector pIBI31 (obtained from International Biotechnology Corp) that contains a promotor for T7 RNA polymerase. Using standard methodologies, an RNA transcript was synthesized encompassing the same sequences used in example 12. This transcript was amplified using standard conditions with all 20 primers in DMG buffer. Taq digested mp18 DNA was used as a control. As seen in Figure 14 there was extensive synthesis. There are characteristic bands that appear in lane 4, the reaction with the RNA template, as well as in lane 2,

the reaction with the DNA template that do not appear in the template independent synthesis seen in lanes 1 and 5.

This demonstrates that the system is capable of utilizing the RNA transcript as a template without the introduction of any other enzyme besides the Klenow, thus proving that the Klenow enzyme can be used as a reverse transcriptase as indicated in the disclosure. This result was studied further by making a Southern blot of the gel seen in Figure 14 and probing with nick-translated biotinylated mp18 DNA using a nick translation kit obtained from Enzo Biochem, Inc. As seen in Figure 15, there was little or no hybridization of the probe to the reaction product of the template independent reactions (lanes 1 and 5) whereas extensive hybridization was observed with the RNA derived reaction (lane 4) as well as the DNA template derived reaction (lane 2), as expected.

Example 14

Strand Displacement Using Ethidium-Labeled Oligonucleotides

Three oligonucleotides with the sequence listed in Figure 16 were prepared and labeled F1, F1C and F3. The unlabeled complement of F1C was hybridized to unlabeled F1. The ratio of F1C: F1 for the hybridization was 1:2. (F1C at a concentration of 0.13 O.D/ml and F1 at a concentration of 0.26 O.D./ml.) Hybridization was performed in 1X SSC for two hours at 45°C.

Aliquots of the hybrid were mixed with different amounts of ethidium-labeled F1 (F1E) in 1X SSC and incubated for 18 hours either at 43°C. or at 37°C. The ratios of F1E oligo to the unlabeled oligo F1C was 1:1, 2:1, 3:1 and 4:1. (The 1:1 reaction contained 0.0325 O.D of the F1E, 0.065 O.D. of F1 and 0.0325 O.D. of F1C.) At the end of the incubation period, 50µl of each mixture was incubated with 50µl of diazonium mixture for 5 minutes at room temperature. To prepare the diazonium mixture, 10 µl of the diazonium stock solution, (50 mM in 1M HCl), was added to 100 µl of cold dilution buffer, (1 X SSC and 0.2 M $KHCO_3$, prepared fresh). The diazonium stock solution is stored at -20°C.

Under these conditions the diazonium will destroy the fluorescence associated with the ethidium in single stranded oligonucleotides. See, e.g., European Patent Application Publication No. 0 492 570 A1, published on July 1, 1992, based on priority document, U.S. Patent Application Serial No. 07/633,730, filed on December 24, 1990, the contents of which are incorporated by reference. But the diazonium will not destroy the fluorescence associated with the ethidium that has intercalated into the double stranded DNA. The survival of the ethidium, under these reaction conditions, is a measure of the extent of formation of a double helix by the ethidium-labeled oligonucleotides, thus indicating displacement of the non-ethidium containing strand by that of the ethidium labeled. This property of the ethidium labeled oligonucleotides by primers can be usefully employed to facilitate initiation of polymerization on double stranded templates. As seen in the figure in Figure 17, the ethidium-labeled oligo displaces the non-ethidium-labeled oligo better at 43°C than at 37°C.

Example 15

T7 Promoter Oligonucleotide 50 Mer Labeled with Ethidium

An oligonucleotide 50-mer including the T7 promoter region of IBI 31 plasmid constructs (plasmid sequences derived from manufacturer, International Biotechnology, Inc.) was synthesized. Its sequence is as follows:

3'-TAC T*AA T*GC GGT* CT*A T*AG T*T--AA TCA TGA AT--T AAT* TAT* GCT* GAG T*GA T*AT* C-5',

where T* represents allylamine dU, and therefore ethidium modification and the 10 base sequence set off by dashes (--AA TCA TGA AT--) was introduced to provide a restriction enzyme site.

Example 16

Use of the Oligonucleotide 50-Mer to Regulate RNA Synthesis *In Vitro*

This nucleotide is complementary to the ATG strand of the lac z gene of IBI 31, and also contains a 10-base sequence for use in restriction enzyme digestion. The oligonucleotide 50-mer also contains sequences overlapping the T7 promotor in the IBI 31 plasmid constructs. Thus, it might be expected to interfere with *in vitro* transcription by T7 RNA polymerase even though the sequences in this oligo are entirely upstream of the start of

transcription by T7 RNA polymerase. Because the plasmid constructs contain opposing T7 and T3 promotors, this also means that the oligo 50-mer is identical in sequence to the RNA that is made by the T3 RNA polymerase *in vitro*.

The effect of this oligonucleotide on *in vitro* transcription by T7 and T3 polymerases from an IBI 31 plasmid construct (pIBI 31-BH5-2) and from a BlueScript II plasmid construct (pBSII/HCV) was studied. See Figure 18 which contains the same target sequences, but in a "split" arrangement. The polylinker sequences of these plasmids are given in Figure 18. Comparing the effect of the oligo on these two different target template serves to partially control for the possible non-specific inhibitory effects of ethidium groups on the RNA polymerases because the oligonucleotide would be expected to inhibit transcription from any template containing an appropriate promotor regardless of the "split" if the effect were due to the oligo's interaction with the polymerase rather than with the template.

At a concentration of 60-fold excess of oligonucleotide (0.6μM final) over plasmid with either the allylamine labelled oligonucleotide of the ethidium labelled oligonucleotide in a transcription reaction mixture, the following results were obtained:

Plasmid Transcribed	Polymerase Used	Oligo Used	nanomoles Incorporated	% of control
pIBI 31-BH5-2	T3	None	236	100
pIBI 31-BH5-2	T3	Allylamine labeled	233	99
pIBI 31-BH5-2	T3	Ethidium labeled	87	37
pIBI 31-BH5-2	T7	None	208	100
pIBI 31-BH5-2	T7	Allylamine labeled	198	95
pIBI 31BH-5-2	T7	Ethidium labeled	3	1.4
pBSII/HCV	T3	None	112	100
pBSII/HCV	T3	Allylamine labeled	158	>100
pBSII/HCV	T3	Ethidium labeled	185	>100
pBSII/HCV	T7	None	125	100
pBSII/HCV	T7	Allylamine labeled	154	>100
pBSII/HCV	T7	Ethidium labeled	62	50

These results indicate that the ethidium-modified oligo sequence is capable of specifically inhibiting transcription by the T7 polymerase from the T7 promotor region provided that the promoter region is not interrupted by the multiple cloning region and inserted DNA. Thus, the effect is dependent on the template DNA and is not merely the result of inhibition of the T7 polymerase by the ethidium groups.

Many obvious variations will be suggested to those of ordinary skill in the art in light of the above detailed description of the invention. All such variations are fully embraced by the scope and spirit of the present invention as set forth in the claims which follow.

WHAT IS CLAIMED IS:

1. An *in vitro* process for producing more than one copy of a specific nucleic acid, said process being independent of a requirement for the introduction of an intermediate structure for the production of said specific nucleic acid, said process comprising the steps of:

(a) providing a nucleic acid sample containing or suspected of containing the sequence of said specific nucleic acid;

(b) contacting said sample with a mixture comprising:

(i) nucleic acid precursors,

(ii) one or more specific nucleic acid primers each of which is complementary to a distinct sequence of said specific nucleic acid, and

(iii) an effective amount of a nucleic acid producing catalyst; and

(c) allowing said mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing more than one copy of said specific nucleic acid.

2. The process of claim 1 wherein said specific nucleic acid is single-stranded or double-stranded.

3. The process of claim 1 wherein said specific nucleic acid is selected from deoxyribonucleic acid, ribonucleic acid, a DNA.RNA hybrid or a polymer capable of acting as a template for a nucleic acid polymerizing catalyst.

4. The process of claim 1 wherein said specific nucleic acid is in solution.

5. The process of claim 4 further comprising the step of treating said specific nucleic acid with a blunt-end promoting restriction enzyme.

6. The process of claim 1 wherein said specific nucleic acid is isolated or purified prior to the contacting step (b) or the reacting step (c).

7. The process of claim 6 wherein said isolation or purification of said specific nucleic acid is carried out by means of sandwich or sandwich capture.

8. The process of claim 7 further comprising the step of releasing said captured specific nucleic acid.

9. The process of claim 8 wherein said releasing step is carried out by means of a restriction enzyme.

10. The process of claim 1 wherein said nucleic acid precursors are selected from nucleoside triphosphates and nucleoside trisphosphate analogs, or a combination thereof.

11. The process of claim 10 wherein said nucleoside triphosphates are selected from deoxyadenosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, deoxythymidine 5'-triphosphate, deoxycytidine 5'-triphosphate, adenosine 5'-triphosphate, guanosine 5'-triphosphate, uridine 5'-triphosphate and cytidine 5'-triphosphate, or a combination of any of the foregoing.

12. The process of claim 10 wherein said nucleoside triphosphate analogs are naturally occurring or synthetic, or a combination thereof.

13. The process of claim 10 wherein at least one of said nucleoside triphosphates or nucleoside triphosphate analogs is modified on the sugar, phosphate or base.

14. The process of claim 1 wherein said specific nucleic acid primers are selected from deoxyribonucleic acid, ribonucleic acid, a DNA.RNA copolymer, or a polymer capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization.

15. The process of claim 1 wherein said specific nucleic acid primers comprise oligo- or polynucleotides.

16. The process of claim 1 wherein said specific nucleic acid primers contain a 3'-hydroxyl group or an isosteric configuration of heteroatoms.

17. The process of claim 16 wherein said heteroatoms are selected from nitrogen, sulfur, or both.

18. The process of claim 1 wherein said specific nucleic acid primers are not substantially complementary to one another.

19. The process of claim 18 wherein said specific nucleic acid primers contain no more than five complementary base-pairs in the sequences therein.

20. The process of claim 1 wherein said specific nucleic acid primers comprise from about 5 to about 100 nucleotides.

21. The process of claim 20 wherein said specific nucleic acid primers comprise from about 8 to about 20 nucleotides.

22. The process of claim 1 wherein said specific nucleic acid primers comprise at least one non-complementary nucleotide or nucleotide analog base, or at least one sequence thereof.

23. The process of claim 22 wherein said specific nucleic acid primers further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs.

24. The process of claim 23 wherein said noncomplementary nucleotide or nucleotide analogs in said specific nucleic acid primers comprise from about 5 to about 20 nucleotides.

25. The process of claim 22 wherein said noncomplementary base sequence or sequences are linked together by other than a phosphodiester bond.

26. The process of claim 1 wherein said nucleic acid producing catalyst is selected from DNA polymerase and reverse transcriptase, or both.

27. The process of claim 1 wherein said nucleic precursors or said specific primers or both are modified by at least one intercalating agent.

28. The process of claim 1 further comprising the step (d) of detecting the product produced in step (c).

29. The process of claim 28 wherein said detecting step (d) is carried out by means of incorporating into the product a labeled primer, a labeled precursor, or a combination thereof.

30. The process of claim 1 further comprising the step of regenerating said one or more specific nucleic acid primers.

31. An *in vitro* process for producing more than one copy of a specific nucleic acid, said products being substantially free of any primer-coded sequences, said process comprising the steps of:

(a) providing a nucleic acid sample containing or suspected of containing the sequence of said specific nucleic acid;

(b) contacting said sample with a mixture comprising:

(i) nucleic acid precursors,

(ii) one or more specific polynucleotide primers comprising at least one ribonucleic acid segment each of which primer is substantially complementary to a distinct sequence of said specific nucleic acid, and

(iii) an effective amount of a nucleic acid producing catalyst; and

(c) allowing said mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing at least one copy of said specific nucleic acid; and

(d) removing substantially or all primer-coded sequences from the product produced in step (c) to regenerate a primer binding site, thereby allowing a new priming event to occur and producing more than one copy of said specific nucleic acid.

32. The process of claim 31 wherein said step (d) removing is carried by digestion with an enzyme.

33. The process of claim 32 wherein said enzyme comprises ribonuclease H.

34. The process of claim 31 wherein said nucleic acid precursors are modified or unmodified.

35. The process of claim 31 wherein said specific polynucleotide primers further comprise deoxyribonucleic acid.

36. The process of claim 31 wherein said specific polynucleotide primers contain a 3'-hydroxyl group or an isosteric configuration of heteroatoms.

37. The process of claim 36 wherein said heteroatoms are selected from nitrogen, sulfur, or both.

38. The process of claim 31 wherein said specific polynucleotide primers further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs.

39. An *in vitro* process for producing more than one copy of a specific nucleic acid, said products being substantially free of any primer-coded sequences, said process comprising the steps of:

(a) providing a nucleic acid sample containing or suspected of containing the sequence of said specific nucleic acid;

(b) contacting said sample with a mixture comprising:

(i) unmodified nucleic acid precursors,

(ii) one or more specific chemically-modified primers each of which primer is substantially complementary to a distinct sequence of said specific nucleic acid, and

(iii) an effective amount of a nucleic acid producing catalyst; and

(c) allowing said mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing at least one copy of said specific nucleic acid; and

(d) removing substantially or all primer-coded sequences from the product produced in step (c) to regenerate a primer binding site, thereby allowing a new priming event to occur and producing more than one copy of said specific nucleic acid.

40. The process of claim 39 wherein said step (d) removing is carried by digestion with an enzyme.

41. The process of claim 40 wherein said enzyme comprises ribonuclease H.

42. The process of claim 39 wherein said specific chemically modified primers are selected from ribonucleic acid, deoxyribonucleic acid, a DNA.RNA copolymer, and a polymer capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization, or a combination of any of the foregoing.

43. The process of claim 39 wherein said specific chemically modified primers contain a 3'-hydroxyl group or an isosteric configuration of heteroatoms.

44. The process of claim 43 wherein said heteroatoms are selected from nitrogen, sulfur, or both.

45. The process of claim 39 wherein said specific chemically modified primers are selected from nucleoside triphosphates and nucleoside triphosphate analogs, or a combination thereof, wherein at least one of said nucleoside triphosphates or analogs is modified on the sugar, phosphate or base.

46. The process of claim 39 wherein said specific chemically modified primers further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs.

47. An *in vitro* process for producing more than one copy of a specific nucleic acid, said products being substantially free of any primer-coded sequences, said process comprising the steps of:

(a) providing a nucleic acid sample containing or suspected of containing the sequence of said specific nucleic acid;

(b) contacting said sample with a mixture comprising:

(i) unmodified nucleic acid precursors,

(ii) one or more specific unmodified primers comprising at least onesegment each of which primer comprises at least one non-complementary sequence to a distinct sequence of said specific nucleic acid, such that upon hybridization to said specific nucleic acid at least one loop structure is formed, and

(iii) an effective amount of a nucleic acid producing catalyst; and

(c) allowing said mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing at least one copy of said specific nucleic acid; and

(d) removing substantially or all primer-coded sequences from the product produced in step (c) to regenerate a primer binding site, thereby allowing a new priming event to occur and producing more than one copy of said specific nucleic acid.

48. The process of claim 47 wherein said step (d) removing is carried by digestion with an enzyme.

49. The process of claim 48 wherein said enzyme comprises ribonuclease H.

50. The process of claim 47 wherein said specific unmodified primers are selected from ribonucleic acid, deoxyribonucleic acid, a DNA.RNA copolymer, and a polymer capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization, or a combination of any of the foregoing.

51. The process of claim 47 wherein said specific unmodified primers further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs.

52. A promoter-independent non-naturally occurring nucleic acid construct which when present in a cell produces a nucleic acid without the use of any gene product coded by said construct.

53. The construct of claim 52 comprising double-stranded and single-stranded nucleic acid regions.

54. The construct of claim 52 wherein said nucleic acid comprises deoxyribonucleic acid, ribonucleic acid, a DNA.RNA copolymer, or a polymer capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization.

55. The construct of claim 52 comprising at least one modified nucleotide or nucleotide analog.

56. The construct of claim 52 comprising at least one single-stranded region.

57. The construct of claim 56 wherein said single-stranded region comprises a bubble.

58. The construct of claim 57 wherein said bubble comprises at least one complementary sequence to a nucleic acid present in the cell.

59. The construct of claim 57 wherein said bubble comprises at least one polyT sequence.

60. A conjugate comprising a protein-nucleic acid construct, said nucleic acid construct not coding for said protein, and which conjugate produces a nucleic acid when present in a cell.

61. The conjugate of claim 60 wherein said protein comprises an RNA polymerase or a subunit thereof and the nucleic acid construct contains the corresponding RNA polymerase promoter.

62. The conjugate of claim 61 wherein said RNA polymerase is selected from T7, T3 and SP6, or a combination of any of the foregoing.

63. The conjugate of claim 60 wherein said protein comprises DNA polymerase or reverse transcriptase and said nucleic acid construct contains at least one sequence complementary to an RNA molecule.

64. The conjugate of claim 60 wherein said nucleic acid construct is double-stranded, single-stranded, or partially single-stranded.

65. The conjugate of claim 60 wherein said nucleic acid construct comprises at least one chemically modified nucleotide or nucleotide analog.

66. The conjugate of claim 60 wherein said protein is linked to said nucleic acid construct by means of a covalent linkage.

67. The conjugate of claim 60 wherein said protein is linked to said nucleic acid construct by means of base-pairing of complementary nucleic acid sequences.

68. The conjugate of claim 60 wherein said protein is linked to said nucleic acid construct by means of a nucleic acid binding protein.

69. The conjugate of claim 68 wherein said nucleic acid binding protein comprises a repressor protein bound to an enzyme.

70. The conjugate of claim 60 wherein said protein is linked to said nucleic acid construct by means of ligand receptor binding.

71. The conjugate of claim 60 wherein the nucleic acid produced is deoxyribonucleic acid, ribonucleic acid, or a combination thereof.

72. The conjugate of claim 60 wherein the nucleic acid produced is sense or antisense, or both.

73. An *in vivo* process for producing a specific nucleic acid, said process comprising the steps of:

(a) providing a conjugate comprising a protein-nucleic acid construct, said conjugate being capable of producing a nucleic acid when present in a cell; and

(b) introducing said conjugate into a cell, thereby producing said specific nucleic acid.

74. The process of claim 73 wherein said construct comprises at least one promoter.

75. The process of claim 73 wherein said construct comprises at least one complementary sequence to a primer present in the cell.

76. The process of claim 73 wherein said nucleic acid construct codes for the protein in said conjugate.

77. The process of claim 73 wherein said nucleic acid construct codes for a protein other than the protein in said conjugate.

78. The process of claim 77 wherein said other protein comprises a nucleic acid polymerase.

79. The process of claim 78 wherein said polymerase comprises an RNA polymerase and said nucleic acid construct comprises a promoter for said RNA polymerase.

80. The process of claim 78 wherein said polymerase comprises a DNA polymerase or reverse transcriptase.

81. A construct comprising a host promoter located on the construct such that the host transcribes a sequence in the construct coding for a different RNA polymerase which after translation is capable of recognizing its cognate promoter and transcribing from a DNA sequence of interest in the construct with said cognate promoter oriented such that it does not promote transcription from the construct of said different RNA polymerase.

82. The construct of claim 81 wherein said host promoter comprises a prokaryotic or eukaryotic promoter upstream from the host promoter.

83. The construct of claim 81 wherein said host promoter and the promoter for the second RNA polymerase are located on opposite strands.

84. The construct of claim 82 wherein said prokaryotic promoter comprises a RNA polymerase.

85. The construct of claim 82 wherein said eukaryotic promoter is selected from Pol I, Pol II and Pol III, or a combination of any of the foregoing.

86. The construct of claim 81 wherein said second RNA polymerase is selected from T7, T3 and SP6, or a combination of any of the foregoing.

87. The construct of claim 81 wherein said DNA sequence of interest comprises sense or antisense, or both.

88. The construct of claim 81 wherein said DNA sequence of interest comprises deoxyribonucleic acid or ribonucleic acid.

89. The construct of claim 81 wherein said DNA sequence of interest encodes a protein.

90. The construct of claim 81 comprising at least one chemically modified nucleotide.

* * * * * * *

ABSTRACT OF THE DISCLOSURE

NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES

This invention provides *inter alia* an *in vitro* process for producing multiple specific nucleic acid copies in which the copies are produced under isostatic conditions, e.g., temperature, buffer and ionic strength, and independently of any requirement for introducing an intermediate structure for producing the copies. In other aspects, the invention provides *in vitro* processes for producing multiple specific nucleic acid copies in which the products are substantially free of any primer-coded sequences, such sequences having been substantially or all removed from the product to regenerate a primer binding site, thereby allowing new priming events to occur and multiple nucleic acid copies to be produced. This invention further provides a promoter-independent non-naturally occurring nucleic acid construct that produces a nucleic acid copy or copies without using or relying on any gene product that may be coded by the nucleic acid construct. Another aspect of this invention concerns a protein-nucleic acid construct in the form of a conjugate linked variously, e.g., covalent linkage, complementary nucleic acid base-pairing, nucleic acid binding proteins, or ligand receptor binding. Further disclosed in this invention is an *in vivo* process for producing a specific nucleic acid in which such a protein-nucleic acid construct conjugate is introduced into a cell. A still further aspect of the invention relates to a construct comprising a host promoter, second promoter and DNA sequence uniquely located on

the construct. The host transcribes a sequence in the construct coding for a different RNA polymerase which after translation is capable of recognizing its cognate promoter and transcribing from a DNA sequence of interest in the construct with the cognate promoter oriented such that it does not promote transcription from the construct of the different RNA polymerase.

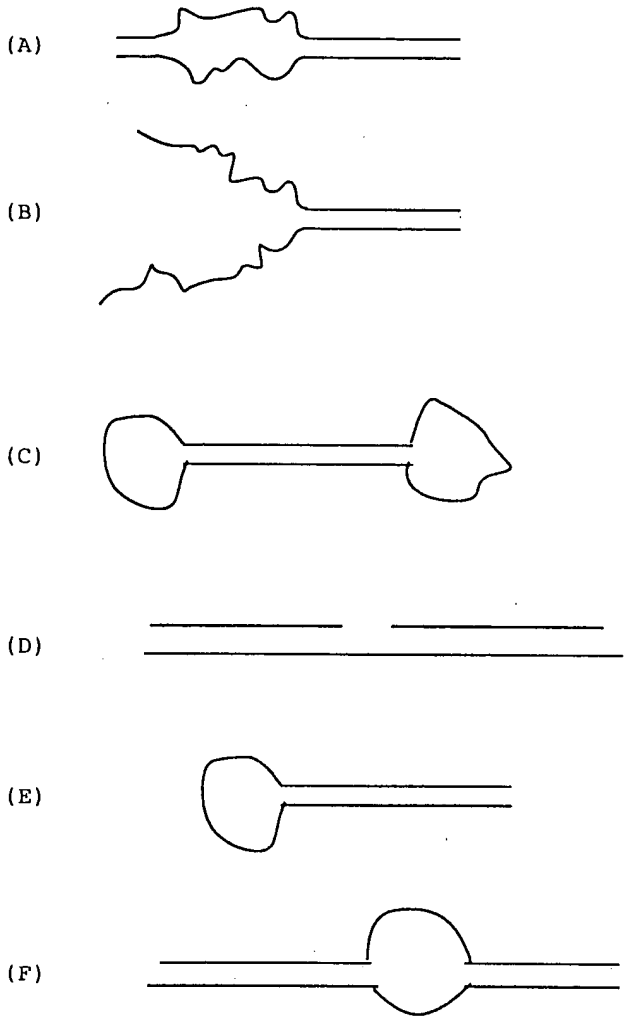


Figure 1 (A-F)

Construct Forms Comprising at Least one Single-Stranded Region

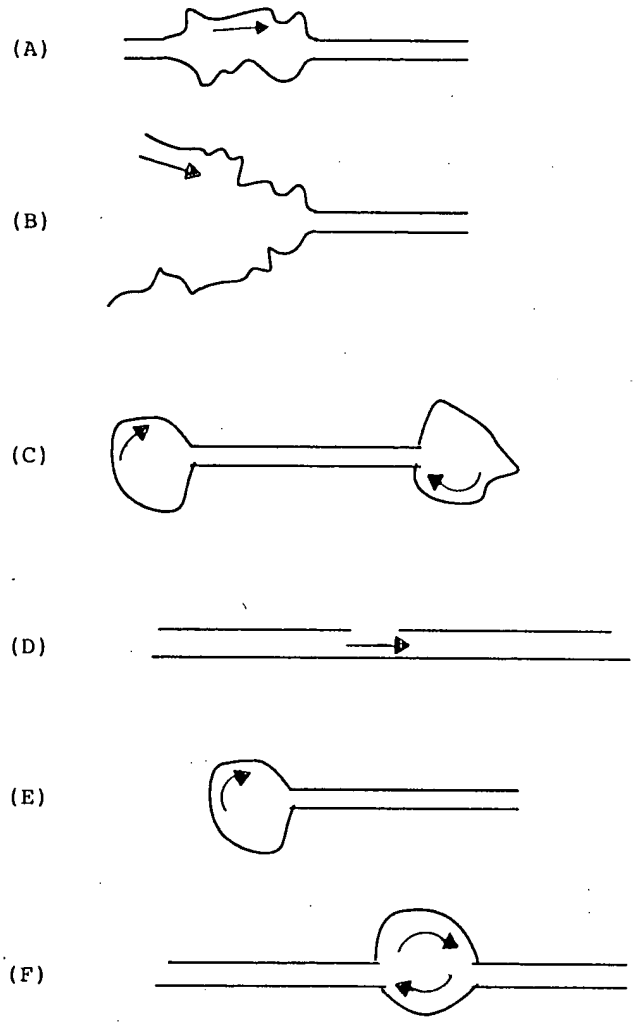


Figure 2 (A-F)

Functional Forms of the Construct

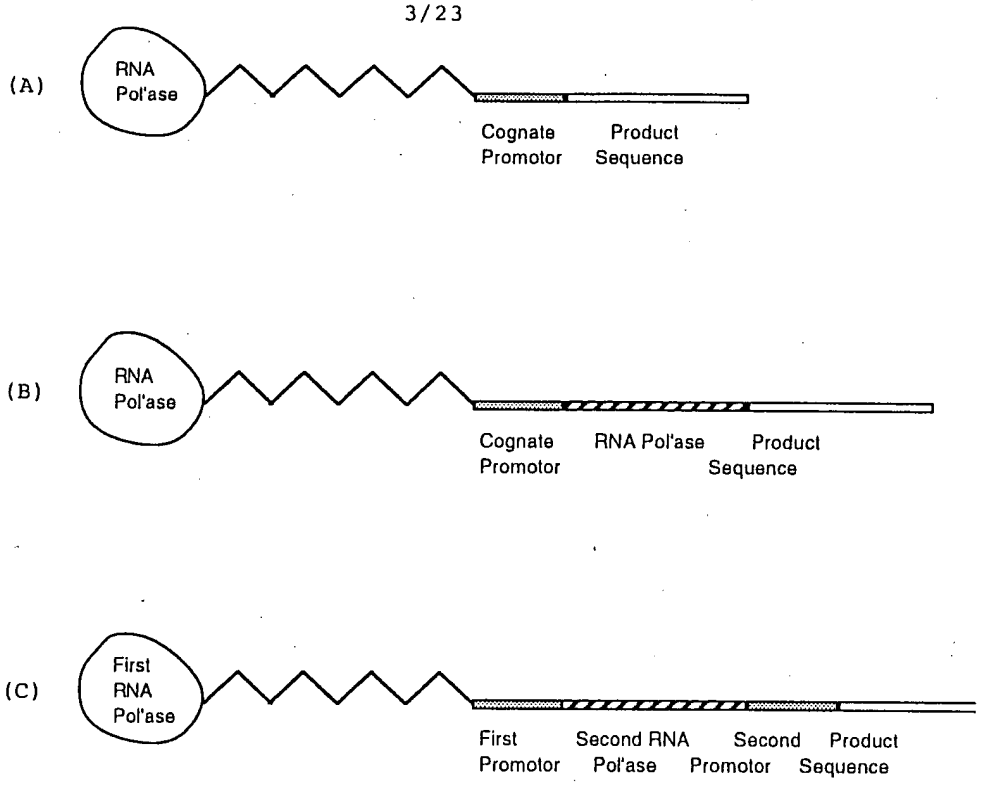


Figure 3 (A-C)

Three Constructs with an RNA Polymerase Covalently Attached to a Transcribing Cassette

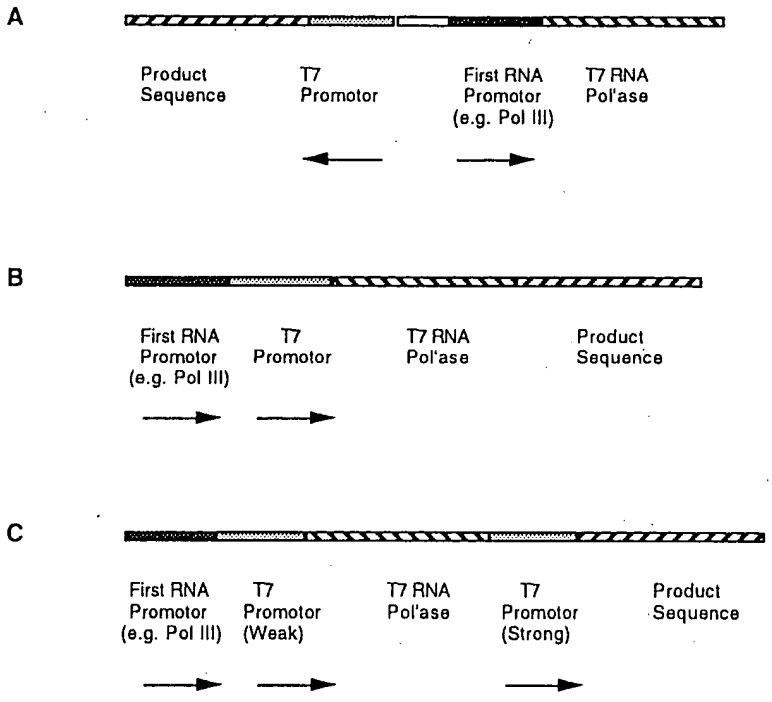


Figure 4 (A-C)

Three Constructs with Promoters for Endogenous RNA Polymerase

M13mp18. Seq Length: 7250

1.	AATGCTACTA	CTATTAGTAG	AATTGATGCC	ACCTTTTCAG	CTCGCGCCCC
51.	AAATGAAAAT	ATAGCTAAAC	AGGTTATTGA	CCATTTGCGA	AATGTATCTA
101.	ATGGTCAAAC	TAAATCTACT	CGTTCGCAGA	ATTGGGAATC	AACTGTTACA
151.	TGGAATGAAA	CTTCCAGACA	CCGTACTTTA	GTTGCATATT	TAAAACATGT
201	TGAGCTACAG	CACCAGATTC	AGCAATTAAG	CTCTAAGCCA	TCCGCAAAAA
251	TGACCTCTTA	TCAAAAGGAG	CAATTAAAGG	TACTCTCTAA	TCCTGACCTG
301.	TTGGAGTTTG	CTTCCGGTCT	GGTTCGCTTT	GAAGCTCGAA	TTAAAACGCG
351.	ATATTTGAAG	TCTTTCGGGC	TTCCTCTTAA	TCTTTTTGAT	GCAATCCGCT
401.	TTGCTTCTGA	CTATAATAGT	CAGGGTAAAG	ACCTGATTTT	TGATTTATGG
451.	TCATTCTCGT	TTTCTGAACT	GTTTAAAGCA	TTTGAGGGGG	ATTCAATGAA
501.	TATTTATGAC	GATTCCGCAG	TATTGGACGC	TATCCAGTCT	AAACATTTTA
551.	CTATTACCCC	CTCTGGCAAA	ACTTCTTTTG	CAAAAGCCTC	TCGCTATTTT
601.	GGTTTTTATC	GTCGTCTGGT	AAACGAGGGT	TATGATAGTG	TTGCTCTTAC
651.	TATGCCTCGT	AATTCCTTTT	GGCGTTATGT	ATCTGCATTA	GTTGAATGTG
701.	GTATTCCTAA	ATCTCAACTG	ATGAATCTTT	CTACCTGTAA	TAATGTTGTT
751.	CCGTTAGTTC	GTTTTATTAA	CGTAGATTTT	TCTTCCCAAC	GTCCTGACTG
801.	GTATAATGAG	CCAGTTCTTA	AAATCGCATA	AGGTAATTCA	CAATGATTAA
851.	AGTTGAAATT	AAACCATCTC	AAGCCCAATT	TACTACTCGT	TCTGGTGTTC
901.	TCGTCAGGGC	AAGCTTATT	CACTGAATGA	GCAGCTTTGT	TACGTTGATT
951.	TGGGTAATGA	ATATCCGGTT	CTTGTCGAAG	ATTACTCTTG	ATGAAGGTCA
1001	GCCAGCCTAT	GCGCCTGGTC	TGTACACCGT	TCATCTGTCC	TCTTTCAAAG
1051	TTGGTCAGTT	CGGTTCCCTT	ATGATTGACC	GTCTGCGCCT	CGTTCCGGCT
1101	AAGTAACATG	GAGCAGGTCG	CGGATTTCGA	CACAATTTAT	CAGGCGATGA
1151	TACAAATCTC	CGTTGTACCTT	TGTTTCGCGC	TTGGTATAAT	CGCTGGGGGT
1201	CAAAGATGAG	TGTTTTAGTG	TATTCTTTCG	CCTCTTTCGT	TTTAGGTTGG

Figure 5

M13mp18 Nucleic Acid Sequence

1251	TGCCTTCGTA	GTGGCATTAC	GTATTTTACC	CGTTTAATGG	AAACTTCCTC
1301	ATGAAAAAGT	CTTTAGTCCT	CAAAGCCTCT	GTAGCCGTTG	CTACCCTCGT
1351	TCCGATGCTG	TCTTTCGCTG	CTGAGGGTGA	CGATCCCGCA	AAAGCGGCCT
1401	TTAACTCCCT	GCAAGCCTCA	GCGACCGAAT	ATATCGGTTA	TGCGTGGGCG
1451	ATGGTTGTTG	TCATTGTCGG	CGCAACTATC	GGTATCAAGC	TGTTTAAGAA
1501	ATTCACCTCG	AAAGCAAGCT	GATAAACCGA	TACAATTAAA	GGCTCCTTTT
1551	GGAGCCTTTT	TTTTTGGAGA	TTTTCAACGT	GAAAAAATTA	TTATTCGCAA
1601	TTCCTTTAGT	TGTTCCTTTC	TATTCTCACT	CCGCTGAAAC	TGTTGAAAGT
1651	TGTTTAGCAA	AACCCCATAC	AGAAAATTCA	TTTACTAACG	TCTGGAAAGA
1701	CGACAAAACT	TTAGATCGTT	ACGCTAACTA	TGAGGGTTGT	CTGTGGAATG
1751	CTACAGGCGT	TGTAGTTTGT	ACTGGTGACG	AAACTCAGTG	TTACGGTACA
1801	TGGGTTCCTA	TTGGGCTTGC	TATCCCTGAA	AATGAGGGTG	GTGGCTCTGA
1851	GGGTGGCGGT	TCTGAGGGTG	GCGGTTCTGA	GGGTGGCGGT	ACTAAACCTC
1901	CTGAGTACGG	TGATACACCT	ATTCCGGGCT	ATACTTATAT	CAACCCTCTC
1951	GACGGCACTT	ATCCGCCTGG	TACTGAGCAA	AACCCGCTA	ATCCTAATCC
2001	TTCTCTTGAG	GAGTCTCAGC	CTCTTAATAC	TTTCATGTTT	CAGAATAATA
2051	GGTTCCGAAA	TAGGCAGGGG	GCATTAACTG	TTTATACGGC	CACTGTTACT
2101	CAAGGCACTG	ACCCCGTTAA	AACTTATTAC	CAGTACACTC	CTGTATCATC
2151	AAAAGCCATG	TATGACGCTT	ACTGGAACGG	TAAATTCAGA	GACTGCGCTT
2201	CAAGGCACTG	ACCCCGTTAA	AACTTATTAC	CAGTACACTC	CTGTATCATC
2151	AAAAGCCATG	TGCCTCAACC	TCCTGTCAAT	GCTGGCGGCG	GCTCTGGTGG
2201	TCCATTCTGG	CTTTAATCAA	GATCCATTCG	TTTGTGAATA	TCAAGGCCAA
2251	TCGTCTGACC	TGCCTCAACC	TCCTGTCAAT	GCTGGCGGCG	GCTCTGGTGG
2301	TGGTTCTGGT	GGCGGCTCTG	AGGGTGGTGG	CTCTGAGGGT	GGCGGTTCTG
2351	AGGGTGGCGG	CTCTGAGGGA	GGCGGTTCCG	GTGGTGGCTC	TGGTTCCGGT
2401	GATTTTGATT	ATGAAAAGAT	GGCAAACGCT	AATAAGGGGG	CTATGACCGA
2451	AAATGCCGAT	GAAAACGCGC	TACAGTCTGA	CGCTAAAGGC	AAACTTGATT

Figure 5

M13mp18 Nucleic Acid Sequence

2501 CTGTCGCTAC TGATTACGGT GCTGCTATCG ATGGTTTCAT TGGTGACGTT
2551 TCCGGCCTTG CTAATGGTAA TGGTGCTACT GGTGATTTTG CTGGCTCTAA
2601 TTCCCAAATG GCTCAAGTCG GTGACGGTGA TAATTCACCT TTAATGAATA
2651 ATTTCCGTCA ATATTTACCT TCCCTCCCTC AATCGGTTGA ATGTCGCCCT
2701 TTTGTCTTTA GCGCTGGTAA ACCATATGAA TTTTCTATTG ATTGTGACAA
2751 AATAAACTTA TTCCGTGGTG TCTTTGCGTT TCTTTTATAT GTTGCCACCT
2801 TTATGTATGT ATTTTCTACG TTTGCTAACA TACTGCGTAA TAAGGAGTCT
2851 TTATCATGCC AGTTCTTTTG GGTATTCCGT TATTATTGCG TTTCCTCGGT
2901 TTCCTTCTGG TAACTTTGTT CGGCTATCTG CTTACTTTTC TTAAAAAGGG
2951 CTTCGGTAAG ATAGCTATTG CTATTTCATT GTTTCTTGCT CTTATTATTG
3001 GGCTTAACTC AATTCTTGTG GGTTATCTCT CTGATATTAG CGCTCAATTA
3051 CCCTCTGACT TTGTTCAGGG TGTTCAGTTA ATTCTCCCGT CTAATGCGCT
3101 TCCCTGTTTT TATGTTATTC TCTCTGTAAA GGCTGCTATT TTCATTTTTG
3151 ACGTTAAACA AAAAATCGTT TCTTATTTGG ATTGGGATAA ATAATATGGC
3201 TGTTTATTTT GTAACTGGCA AATTAGGCTC TGGAAAGACG CTCGTTAGCG
3251 TTGGTAAGAT TCAGGATAAA ATTGTAGCTG GGTGCAAAAT AGCAACTAAT
3301 CTTGATTTAA GGCTTCAAAA CCTCCCGCAA GTCGGGAGGT TCGCTAAAAC
3351 GCCTCGCGTT CTTAGAATAC CGGATAAGCC TTCTATATCT GATTTGCTTG
3401 CTATTGGGCG CGGTAATGAT TCCTACGAATG AAAATAAAAA CGGCTTGCTT
3451 GTTCTCGATG AGTGCGGTAC TTGGTTTAAT ACCCGTTCTT GGAATGATAA
3501 GGAAAGACAG CCGATTATTG ATTGGTTTCT ACTGCTCGT AAATTAGGAT
3551 GGGATATTAT TTTTCTTGTT CAGGACTTAT CTATTGTTGA TAAACAGGCG
3601 CGTTCTGCAT TAGCTGAACA TGTTGTTTAT TGTCGTCGTC TGGACAGAAT
3651 TACTTTACCT TTTGTCGGTA CTTTATATTC TCTTATTACT GGCTCGAAAA
3701 TGCCTCTGCC TAAATTACAT GTTGGCGTTG TTAAATATGG CGATTCTCAA
3751 TTAAGCCCTA CTGTTGAGCG TTGGCTTTAT ACTGGTAAGA ATTTGTATAA
3801 CGCATATGAT ACTAAACAGG CTTTTTCTAG TAATTATGAT TCCGGTGTTT

Figure 5

M13mp18 Nucleic Acid Sequence

3851	ATTCTTATTT	AACGCCTTAT	TTATCACACG	GTCGGTATTT	CAAACCATTA
3901	AATTTAGGTC	AGAAGATGAA	ATTAACTAAA	ATAATATTGA	AAAAGTTTTC
3951	TCGCGTTCTT	TGTCTTGCGA	TTGGATTTGC	ATCAGCATTT	ACATATAGTT
4001	ATATAACCCA	ACCTAAGCCG	GAGGTTAAAA	AGGTAGTCTC	TCAGACCTAT
4051	GATTTTGATA	AATTCACTAT	TGACTCTTCT	CAGCGTCTTA	ATCTAAGCTA
4101	TCGCTATGTT	TTCAAGGATT	CTAAGGGAAA	ATTAATTAAT	AGCGACGATT
4151	TACAGAAGCA	AGGTTATTCA	CTCACATATA	TTGATTTATG	TACTGTTTCC
4201	ATTAAAAAAG	GTAATTCAAA	TGAAATTGTT	AAATGTAATT	AATTTTGTTT
4251	TCTTGATGTT	TGTTTCATCA	TCTTCTTTTG	CTCAGGTAAT	TGAAATGAAT
4301	AATTCGCCTC	TGCGCGATTT	TGTAACTTGG	TATTCAAAGC	AATCAGGCGA
4351	AATCCGTTATT	GTTTCTCCCG	ATGTAAAAGG	TACTGTTACT	GTATATTCAT
4401	CTGACGTTAA	ACCTGAAAAT	CTACGCAATT	TCTTTATTTC	TGTTTTACGT
4451	GCTAATAATT	TTGATAATGGT	TGGTTCAATT	CCTTCCATAA	TTCAGAAGTA
4501	TAATCCAAAC	AATCAGGATT	ATATTGATGA	ATTGCCATCA	TCTGATAATC
4551	AGGAATATGA	TGATAATTCC	GCTCCTTCTG	GTGGTTTCTT	TGTTCCGCAA
4601	AATGATAATG	TTACTCAAAC	TTTTAAAATT	AATAACGTTC	GGGCAAAGGA
4651	TTTAATACGA	GTTGTCGAAT	TGTTTGTAAA	GTCTAATACT	TCTAAATCCT
4701	CAAATGTATT	ATCTATTGAC	GGCTCTAATC	TATTAGTTGT	TAGTGCTCCT
4751	AAAGATATTT	TAGATAACCT	TCCTCAATTC	CTTTCTACTG	TTGATTTGCC
4801	AACTGACCAG	ATATTGATTG	AGGGTTTGAT	ATTTGAGGTT	CAGCAAGGTG
4851	ATGCTTTAGA	TTTTTCATTT	GCTGCTGGCT	CTCAGCGTGG	CACTGTTGCA
4901	GGCGGTGTTA	ATACTGACCG	CCTCACCTCT	GTTTTATCTT	CTGCTGGTGG
4951	TTCGTTCGGT	ATTTTTAATG	GCGATGTTTT	AGGGCTATCA	GTTCGCGCAT
5001	TAAAGACTAA	TAGCCATTCA	AAAATATTGT	CTGTGCCACG	TATTCTTACG
5051	CTTTCAGGTC	AGAAGGGTTC	TATCTCTGTT	GGCCAGAATG	TCCCTTTTAT
5101	TAAAGACTAA	TAGCCATTCA	AAAATATTGT	CTGTGCCACG	TATTCTTACG
5151	CGATTGAGCG	TCAAAATGTA	GGTATTTCCA	TGAGCGTTTT	TCCTGTTGCA

Figure 5

M13mp18 Nucleic Acid Sequence

5201	ATGGCTGGCG	GTAATATTGT	TCTGGATATT	ACCAGCAAGG	CCGATAGTTT
5251	GAGTTCTCT	ACTCAGGCAA	GTGATGTTAT	TACTAATCAA	AGAAGTATTG
5301	CTACAACGGT	TAATTTGCGT	GATGGACAGA	CTCTTTTACT	CGGTGGCCTC
5351	ACTGATTATA	AAAACACTTC	TCAAGATTCT	GGCGTACCGT	TCCTGTCTAA
5401	AATCCCTTTA	ATCGGCCTCC	TGTTTAGCTC	CCGCTCTGAT	TCCAACGAGG
5451	AAAGCACGTT	ATACGTGCTC	GTCAAAGCAA	CCATAGTACG	CGCCCTGTAG
5501	CGGCGCATTA	AGCGCGGCGG	GTGTGGTGGT	TACGCGCAGC	GTGACCGCTA
5551	CACTTGCCAG	CGCCCTAGCG	CCCGCTCCTT	TCGCTTTCTT	CCCTTCCTTT
5601	CTCGCCACGT	TCGCCGGCTT	TCCCCGTCAA	GCTCTAAATC	GGGGGCTCCC
5651	TTTAGGGTTC	CGATTTAGTG	CTTTACGGCA	CCTCGACCCC	AAAAAACTTG
5701	ATTTGGGTGA	TGGTTCACGT	AGTGGGCCAT	CGCCCTGATA	GACGGTTTTT
5751	CGCCCTTTGA	CGTTGGAGTC	CACGTTCTTT	AATAGTGGAC	TCTTGTTCCA
5801	AACTGGAACA	ACACTCAACC	CTATCTCGGG	CTATTCTTTT	GATTTATAAG
5851	GGATTTTGCC	GATTTCGGAA	CCACCATCAA	ACAGGATTTT	CGCCTGCTGG
5901	GGCAAACCAG	CGTGGACCGC	TTGCTGCAAC	TCTCTCAGGG	CCAGGCGGTG
5951	AAGGGCAATC	AGCTGTTGCC	CGTCTCGCTG	GTGAAAAGAA	AAACCACCCT
6001	GGCGCCCAAT	ACGCAAACCG	CCTCTCCCCG	CGCGTTGGCC	GATTCATTAA
6051	TGCAGCTGGC	ACGACAGGTT	TCCCGACTGG	AAAGCGGGCA	GTGAGCGCAA
6101	CGCAATTAAT	GTGAGTTAGC	TCACTCATTA	GGCACCCCAG	GCTTTACACT
6151	TTATGCTTCC	GGCTCGTATG	TTGTGTGGAA	TTGTGAGCGG	ATAACAATTT
6201	CACACAGGAA	ACAGCTATGA	CCATGATTAC	GAATTCGAGC	TCGGTACCCG
6251	GCGATCCTCT	AGAGTCGACC	TGCAGGCATG	CAAGCTTGGC	ACTGGCCGTC
6301	GTTTTACAAC	GTCGTGACTG	GGAAAACCCT	GGCGTTACCC	AACTTAATCG
6351	CCTTGCAGCA	CAATCCCCTT	TCGCCAGCTG	GCGTAATAGC	GAAGAGGCCC
6401	GCACCGATCG	CCCTTCCCAA	CAGTTGCGCA	GCCTGAATGG	CGAATGGCGC
6451	TTTGCCTGGT	TTCCGGCACC	AGAAGCGGTG	CCGGAAAGCT	GGCTGGAGTG
6501	CGATCTTCCT	GAGGCCGATA	CGGTCGTCGT	CCCCTCAAAC	TGGCAGATGC

Figure 5

M13mp18 Nucleic Acid Sequence

6551	ACGGTTACGA	TGCGCCCATC	TACACCAACG	TAACCTATCC	CATTACGGTC
6601	AATCCGCCGT	TTGTTCCCAC	GGAGAATCCG	ACGGGTTGTT	ACTCGCTCAC
6651	ATTTAATGTT	GATGAAAGCT	GGCTACAGGA	AGGCCAGACG	CGAATTATTT
6701	TTGATGGCGT	TCCTATTGGT	TAAAAAATGA	GCTGATTTAA	CAAAAATTTA
6751	ACGCGAATTT	TAACAAAATA	TTAACGTTTA	CAATTTAAAT	ATTTGCTTAT
6801	ACAATCTTCC	TGTTTTTGGG	GCTTTTCTGA	TTATCAACCG	GGGTACATAT
6851	GATTGACATG	CTAGTTTTAC	GATTACCGTT	CATCGATTCT	CTTGTTTGCT
6901	CCAGACTCTC	AGGCAATGAC	CTGATAGCCT	TTGTAGATCT	CTCAAAAATA
6951	GCTACCCTCT	CCGGCATGAA	TTTATCAGCT	AGAACGGTTG	AATATCATAT
7001	TGATGGTGAT	TTGACTGTCT	CCGGCCTTTC	TCACCCTTTT	GAATCTTTAC
7051	CTACACATTA	CTCAGGCATT	GCATTTAAAA	TATATGAGGG	TTCTAAAAAT
7101	TTTTATCCTT	GCGTTGAAAT	AAAGGCTTCT	CCCGCAAAAG	TATTACAGGG
7151	TCATAATGTT	TTTGGTACAA	CCGATTTAGC	TTTATGCTCT	GAGGCTTTAT

Figure 5

M13mp18 Nucleic Acid Sequence

COMPLEMENTARY TO M_{13}

POSITION	5' * 3'	POSITION	
645	AGCAACACTATCATA	631	$M_{13}/1$
615	ACGACGATAAAAACC	601	$M_{13}/2$
585	TTTTGCAAAAGAAGT	571	$M_{13}/3$
555	AATAGTAAAATGTTT	541	$M_{13}/4$
525	CAATACTGCGGAATG	511	$M_{13}/5$
495	TGAATCCCCCTCAAA	481	$M_{13}/6$
465	AGAAAACGAGAATGA	451	$M_{13}/7$
435	CAGGTCTTTACCCTG	421	$M_{13}/8$
405	AGGAAAGCGGATTGC	391	$M_{13}/9$
375	AGGAAGCCCGAAAGA	361	$M_{13}/10$

COMPLEMENTARY TO SS PHAGE DNA

POSITION	5' * * 3'	POSITION	
351	ATATTTGAAGTCTTT	366	$M_{13}/11$
371	TCTTTTTGATGCAAT	386	$M_{13}/12$
391	CTATAATACTCAGGG	406	$M_{13}/13$
411	TGATTTATGGTCATT	426	$M_{13}/14$
431	GTTTAAAGCATTTGA	446	$M_{13}/15$
451	TATTTATGACGATTC	466	$M_{13}/16$
471	TATCCAGTCTAAACA	486	$M_{13}/17$
491	CTCTGGCAAAACTTC	506	$M_{13}/18$
511	TCGCTATTTTGGTTT	526	$M_{13}/19$
531	AAACGAGGGTTATGA	546	$M_{13}/20$

Figure 6

Primers for Nucleic Acid Production Derived from M13mp18 Sequence

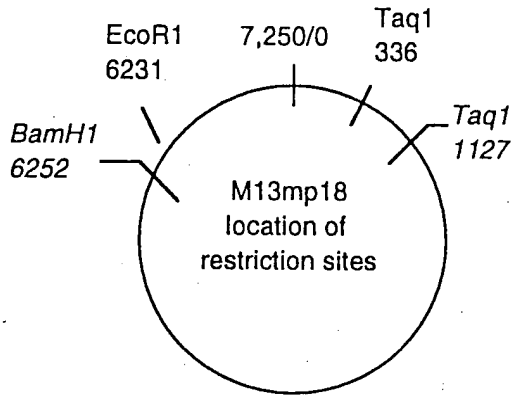


Figure 7

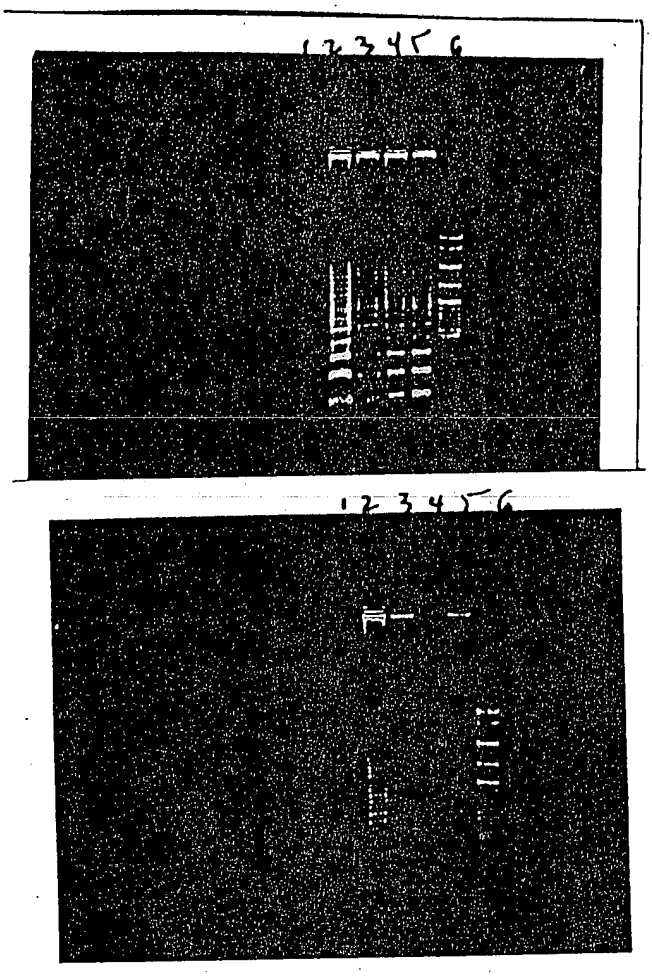
Appropriate M13mp18 Restriction Sites

Lane 1: from calf thymus + Taq digested mp18 amplification reaction
Lane 2: from Taq digested mp18 amplification reaction
Lane 3: from calf thymus amplification reaction
Lane 4: øX174 Hinf1 size marker

Figure 8

Lane 1: no template
Lane 2: mp18 template, phosphate buffer
Lane 3: MspI/pBR322 size marker
Lane 4: mp18 template, MOPS buffer

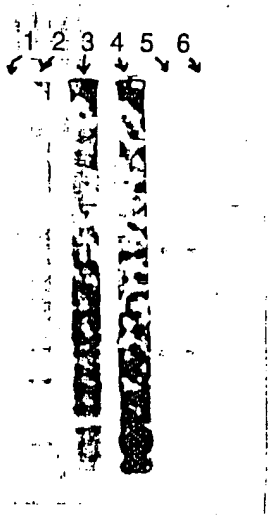
Figure 9



Top= (+) Template
Bottom= (-) Template

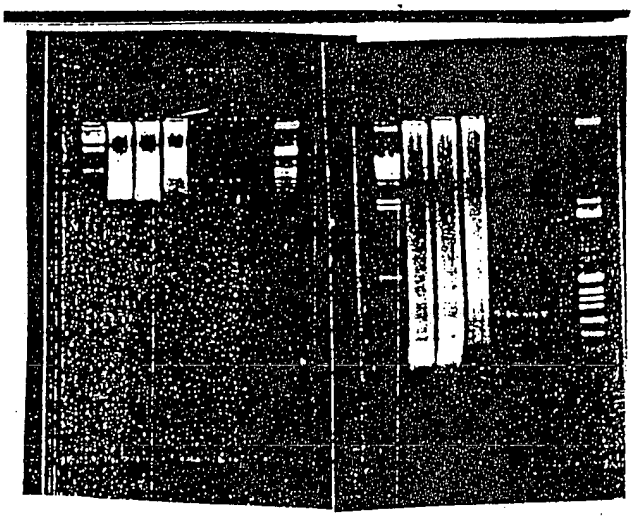
Lane 1: phosphate buffer
Lane 2: MES
Lane 3: MOPS
Lane 4: DMAB
Lane 5: DMG
Lane 6: pBR322/MspI size marker

Figure 10



Lane 1: DMAB buffer, no template
Lane 2: DMAB buffer, mp18 template
Lane 3: DMG buffer, no template
Lane 4: DMG buffer, mp18 template
Lane 5: No reaction
Lane 6: 200 ng Taq I digested mp18 size marker/positive control

Figure 11



First Time Interval Second Time Interval

Agarose Gel Analysis

Lane 1: lambda Hind III marker
Lane 2: Amp/Untreated
Lane 3: Amp/Kinased
Lane 4: Amp/Kinased/Ligated
Lane 5: PCR/Untreated
Lane 6: PCR/Kinased
Lane 7: PCR/Kinased/Ligated
Lane 8: øX174/Hinf1 marker

Figure 12

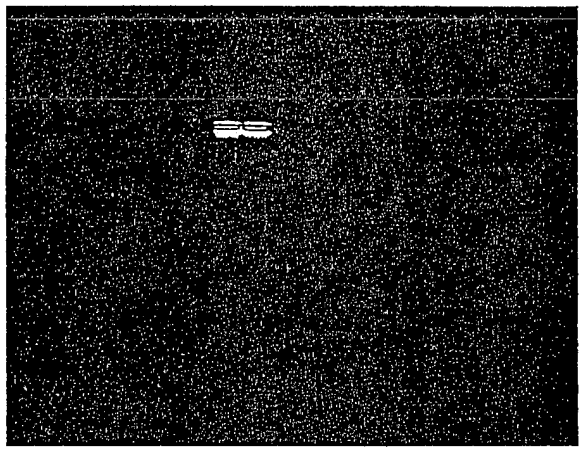
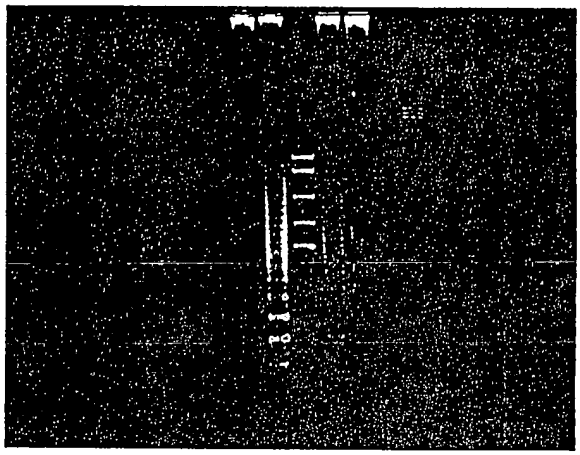


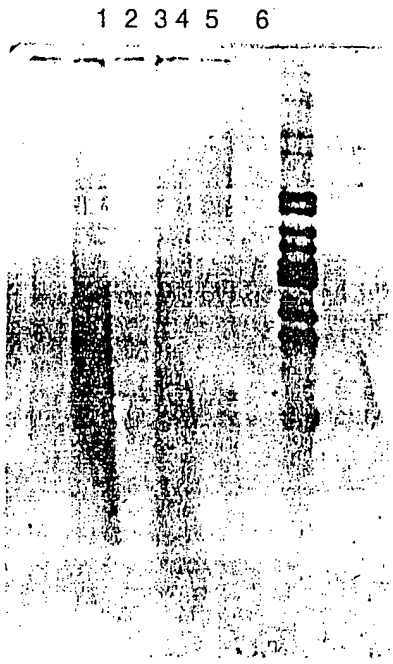
Figure 13

1 2 3 4 5 6



Lane 1: Primers alone
Lane 2: Primers + taq digested M13 DNA
Lane 3: Molecular weight markers
Lane 4: Primers + RNA
Lane 5: Primers alone
Lane 6: M13 digested DNA
Buffer was dimethyl amino glycine, pH 8.6

Figure 14



Lane 1: Primers alone
Lane 2: Primers + taq digested M13 DNA
Lane 3: Molecular weight markers
Lane 4: Primers + RNA
Lane 5: Primers alone
Lane 6: M13 digested DNA
Buffer was dimethyl amino glycine, pH 8.6

Figure 15

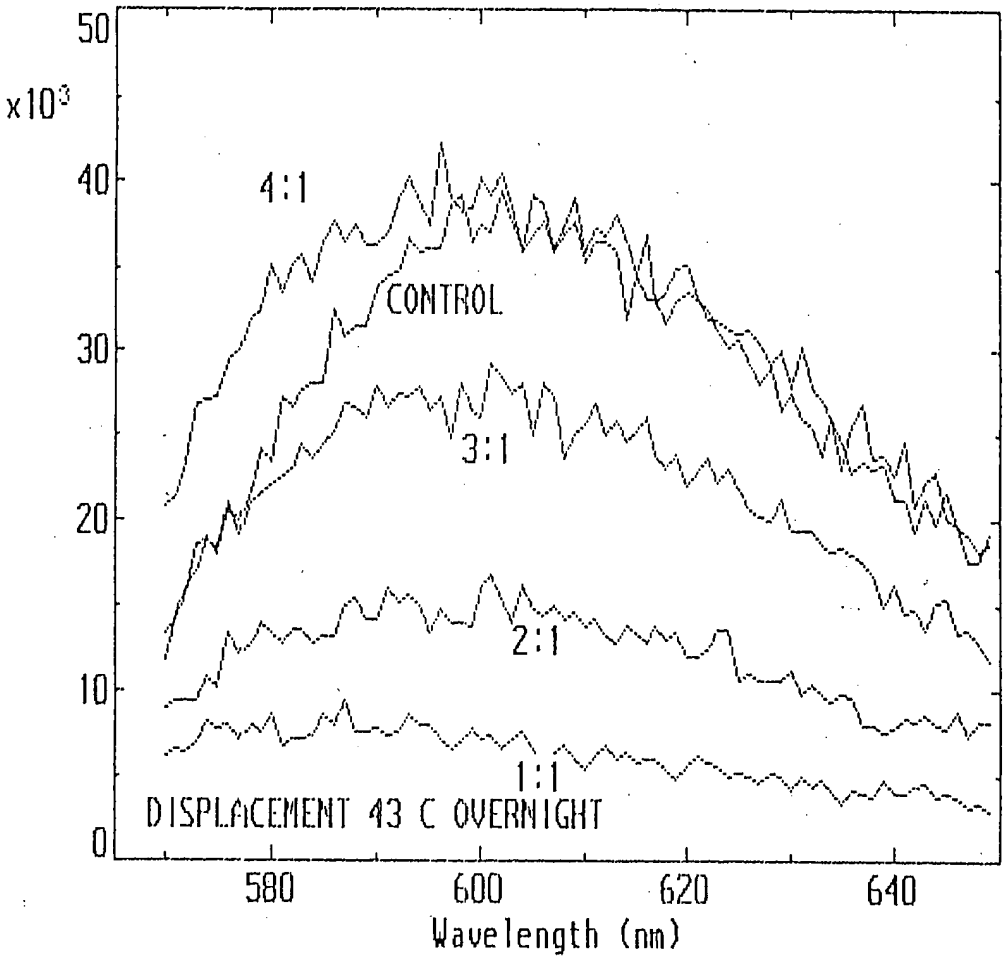

50
x10³
40
30
20
10
0
4:1
CONTROL
3:1
2:1
1:1
DISPLACEMENT 43 C OVERNIGHT
580
600
620
640
Wavelength (nm)

Figure 16

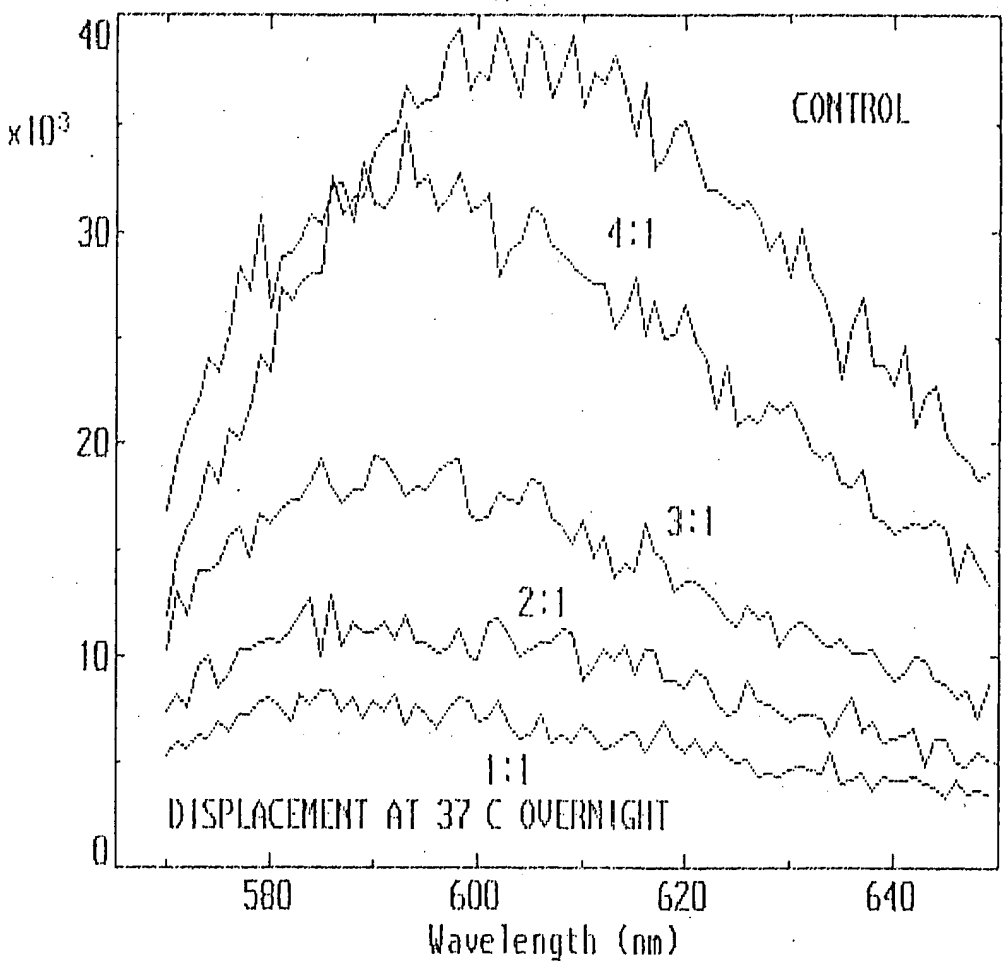

40
30
20
10
0
x10³
CONTROL
4:1
3:1
2:1
1:1
DISPLACEMENT AT 37 C OVERNIGHT
580
600
620
640
Wavelength (nm)

Figure 17

pIBI 31-BH5-2

fmet AUG of Lac z {T7 Promotor region····
LAC PROMOTOR..ATG ACC ATG ATT ACG CCA GAT ATC AAA TTA ATA CGA CTC ACT ATA

oligo 50-mer 3'- tac t*aa t*gc ggt* ct*a t*ag t*Vt aat* tat* gct* gag t*ga t*at* c-5'
10 base insert

T7 RNA Start {«« T3 Promotor Region }
IGGG CTC ICCT TTA GTG ACG GTT AAT
···»»} «- T3 Start Signal

pIBI 31 BSII/HCV

fmet AUG of Lac z {T3 Promotor region -»} T3 RNA Start
LAC PROMOTOR ..ATG ACC ATG ATT ACG CCA AGC TCG AAA TTA ACC CTC ACT AAA /GGG
oligo 50-mer 3'- tac t*aa t*ac t*aa t*gc ggt* t*V--10 base insert--.....

{«- T7 Promotor Region }
MULTIPLE CLONING SITE + 390 BASE INSERT CTA /TAG TGA GTC CGT ATT AAT....
«- T7 Start Signal
5'-ct*a t*ag t*ga gt*c gt*a tt*a at*.....

Figure 18

DECLARATION FOR PATENT APPLICATION

Docket No. ENZ-52

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES

the specification of which

(check one) ___ is attached hereto.
_X_ was filed on **January 13, 1994**, as Application Serial No. **08/182,621**
and was amended on _____, 1994 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United Stated Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §156(a) which occurred between the filing date of the prior application and the national or PCT international filing date of the application:

(Application Serial No.)	(Filing Date)	(Status-patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status-patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Ronald C. Fedus, Esq., Reg. No. 32,567.

Address all telephone calls to: Ronald C. Fedus, Esq., at telephone no. (212)-856-0876
Address all correspondence to: Ronald C. Fedus, Esq., Corporation & Patent Counsel
Enzo Biochem, Inc.
575 Fifth Avenue - 18th Floor
New York, NY 10017

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of ~~sole or first~~ second joint inventor Jannis G. Stavrianopoulos
First Inventor's signature _____ Date _____
Residence 99 South Clinton Avenue (No. 11D), Bay Shore, New York 11706 Citizenship United States
Post Office Address (Same as above)

Full name of ~~second~~ first joint inventor, if any Dean L. Engelhardt
Second Inventor's signature Dean L Engelhardt Date _____
Residence 173 Riverside Drive (No. 6D), New York, New York 10025 Citizenship United States
Post Office Address (Same as above)

Full name of third joint inventor, if any Elazar Rabbani
Third Inventor's signature _____ Date _____
Residence 69 Fifth Avenue (No. 19A) New York, New York 10003 Citizenship United States
Post Office Address (Same as above)

Full name of fourth joint inventor, if any James J. Donegan
Fourth Inventor's signature _____ Date _____
Residence 210 East Broadway (No. 3G) Long Beach, New York 11561 Citizenship United States
Post Office Address (Same as above)

* * * * * * *

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of ~~sole or first~~ second joint inventor Jannis G. Stavrianopoulos
First Inventor's signature _____ Date 6/13/94
Residence 99 South Clinton Avenue (No. 11D), Bay Shore, New York 11706 Citizenship United States
Post Office Address (Same as above)

Full name of ~~second~~ first joint inventor, if any Dean L. Engelhardt
Second Inventor's signature _____ Date _____
Residence 173 Riverside Drive (No. 6D) New York, New York 10025 Citizenship United States
Post Office Address (Same as above)

Full name of third joint inventor, if any Elazar Rabbani
Third Inventor's signature _____ Date _____
Residence 69 Fifth Avenue (No. 19A) New York, New York 10003 Citizenship United States
Post Office Address (Same as above)

Full name of fourth joint inventor, if any James J. Donegan
Fourth Inventor's signature _____ Date 6/13/94
Residence 210 East Broadway (No. 3G) Long Beach, New York 11561 Citizenship United States
Post Office Address (Same as above)

* * * * * * *

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of ~~sole or first~~ second joint inventor Jannis G. Stavrianopoulos
First Inventor's signature _____ Date _____
Residence 99 South Clinton Avenue (No. 11D), Bay Shore, New York 11706 Citizenship United States
Post Office Address (Same as above)

Full name of ~~second~~ first joint inventor, if any Dean L. Engelhardt
Second Inventor's signature _____ Date _____
Residence 173 Riverside Drive (No. 6D) New York, New York 10025 Citizenship United States
Post Office Address (Same as above)

Full name of third joint inventor, if any Elazar Rabbani
Third Inventor's signature _____ Date 11/13/94
Residence 69 Fifth Avenue (No. 19A) New York, New York 10003 Citizenship United States
Post Office Address (Same as above)

Full name of fourth joint inventor, if any James J. Donegan
Fourth Inventor's signature _____ Date _____
Residence 210 East Broadway (No. 3G) Long Beach, New York 11561 Citizenship United States
Post Office Address (Same as above)

* * * * * * *

Docket No.: ENZ-52

ASSIGNMENT

In consideration of One Dollar ($1.00), and other good and valuable consideration, the receipt of which is hereby acknowledged, I , the undersigned,

Dean L. Engelhardt,
Jannis G. Stavrianopoulos,
Elazar Rabbani, and
James J. Donegan,

RESIDING AT:

173 Riverside Drive (No. 6D), New York, New York 10025;
99 South Clinton Avenue (No. 11D), Bay Shore, New York 11706;
69 Fifth Avenue (No. 19A), New York, New York 10003; and
210 East Broadway (No. 3G), Long Beach, New York 11561; respectively

Hereby sell, assign and transfer to: Enzo Diagnostics, Inc., a corporation of the State of New York, having a principal place of business at: 60 Executive Boulevard, Farmingdale, New York 11735-4716, its successors, assigns and legal representatives, the entire right, title and interest for the United States and all foreign countries, in and to any and all improvements which are disclosed in the application for United States Letters Patent, which has been executed by the undersigned concurrently herewith, and is entitled:

NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES

(said application having been filed on January 13, 1994 and accorded U.S. Patent Application Serial No. 08/182,621)

and in and to said application and all divisional, continuing, substitute, renewal, reissue, and all other applications for Letters Patent which have been or shall be filed in the United States and all foreign countries on any of said improvements; and in and to all original and reissued patents which have been or shall be issued in the United States and all foreign countries on said improvements:

Agree that said Assignee may apply for and receive Letters Patent for said improvements in its own name, and that, when requested, without charge to but at the expense of said Assignee, its successors, assigns and legal representatives, to carry out in good faith the intent and purpose of this assignment, the undersigned will execute all divisional, continuing, substitute, renewal, reissue, and all other patent applications on any and all said improvements; execute all rightful oaths, assignments powers of attorney and other papers, communicate to said Assignee, its successors, assigns, and representatives, all facts known to the undersigned relating to said improvements and the history thereof; and generally do everything possible which said Assignee, its successors, assigns or representatives shall consider desirable for aiding in securing and maintaining proper patent protection for said improvements and for vesting title to said improvements and all applications for patents and all patents on said improvements, in said Assignee, its successors, assigns and legal representatives; and

Covenant with said Assignee, its successors, assigns and legal representatives that no assignment, grant, mortgage, license or other agreement affecting the rights and property herein conveyed has been made to others by the undersigned, and that full right to convey the same as herein expressed is possessed by the undersigned.

Jannis G. Stavrianopoulos	(date)
Dean L. Engelhardt	6/14/94 (date)
Elazar Rabbani	(date)
James J. Donegan	(date)

* * * * * * *

Covenant with said Assignee, its successors, assigns and legal representatives that no assignment, grant, mortgage, license or other agreement affecting the rights and property herein conveyed has been made to others by the undersigned, and that full right to convey the same as herein expressed is possessed by the undersigned.

Jannis G. Stavrianopoulos	6/13/94 (date)
Dean L. Engelhardt	(date)
Elazar Rabbani	(date)
James J. Donegan	6/13/94 (date)

* * * * * * *

Covenant with said Assignee, its successors, assigns and legal representatives that no assignment, grant, mortgage, license or other agreement affecting the rights and property herein conveyed has been made to others by the undersigned, and that full right to convey the same as herein expressed is possessed by the undersigned.

Jannis G. Stavrianopoulos	(date)
Dean L. Engelhardt	(date)
Elazar Rabbani	6/13/1994 (date)
James J. Donegan	(date)

* * * * * * *

Attorney's Docket No.: ENZ-52

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Dean L. Engelhardt et al.

Serial No.: 08/182,621 Group Art Unit: Not Yet Known

Filed: January 13, 1994 Examiner: Not Yet Known

For: NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9 (f) and 1.27 (c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:

[X] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN ENZO DIAGNOSTICS, INC.

ADDRESS OF CONCERN c/o ENZO BIOCHEM, INC.
575 FIFTH AVENUE (18TH FLOOR)
NEW YORK, NEW YORK 10017

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Rule 35, United States Code, in that the number of employees of the concern, including those of its affiliates does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled:

NOVEL PROCESS, CONSTRUCT AND CONJUGATE FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES

by inventor(s)

Dean L. Engelhardt et al.

described in

[ ] the specification filed herewith

[X] Application Serial No. 08/182,621, filed January 13, 1994

[ ] Patent No. _____, issued _____.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).

NAME _____

ADDRESS _____

( ) Individual ( ) Small Business Concern ( ) Nonprofit

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING BARRY W. WEINER

TITLE OF PERSON OTHER THAN OWNER EXECUTIVE VICE PRESIDENT

ADDRESS OF PERSON SIGNING 575 FIFTH AVENUE (18TH FLOOR)
NEW YORK, NEW YORK 10017

SIGNATURE Barry W. Weiner DATE JUNE 14, 1994